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STRUCTURE IN RELATION TO CELLULAR FUNCTION

BY

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STRUCTURE IN RELATION TO CELLULAR FUNCTION*

Conference Chairman: ROBERT CHAMBERS

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THE CELL AS AN INTEGRATED FUNCTIONAL BODY

By Robert Chambers
Department of Biology, New York University, New York, N. Y.

The title of this monograph refers to a subject which has been a problem for consideration ever since the protoplasmic body was realized to be the physical basis of the life-giving properties of a cell. It was surmised early that protoplasm with its varied activities could not be a homogeneous substance, hence the several hypotheses of structure with which we are all familiar. It was not, however, until within recent years, when chemistry has so rapidly evolved into a science of structure, that we have been able to introduce logic into our investigations on the relation of structure to protoplasmic function.

Our endeavor here is to touch on characteristics which are common to cells in general. For this reason, we have avoided certain structures of highly specialized cells, such as of muscle and nerve. The subjects treated may well serve as introductory to separate symposia where each can be discussed in greater detail than is possible here.

An adequate study of protoplasm requires an understanding of many facets. These may best be investigated separately in cells in which certain features are intensified—for example, that of reversible contraction in muscle, of conduction in nerve, of solation and gelation in ameboid cells, *etc.* It will then be necessary to combine all available studies to gain insight into the properties of the protoplasmic body common to all cells.

These remarks are intended to present a general morphological concept of a cell as a whole. More particularly, I shall deal with that part which we are terming its extraneous coat in contrast to the surface film of the enclosed protoplasmic body. Cytolysis is involved only when the protoplasmic surface film is broken. Within the protoplasm are regional differentiations which must be considered as intimately related to protoplasmic structure as a whole. Finally, we come to cell division, a property common to plants and animals, an understanding of which process requires examination of their similarities.

It is well known that the earliest cell to be recognized was the plant cell and that the original definition was that a cell consists of a wall surrounding a cavity. We might do well to return to this with the addition introduced by von Mohl, that the cavity contains a body of protoplasm. Purkinje confused the issue by referring to the animal cell as being synonymous with protoplasm. It was not till Curt Herbst presented evidence for an enveloping material, by means of which neighboring cells are stuck together and which can be dissipated in Ca-free media, that we were able to bring into harmony the original concept with that of the present. Normally, the protoplasm of a cell is not naked, but is covered with extraneous coats of varying rigidity. It is these coats which we regard as supporting walls. In animal cells, there is evidence that these coatings are the sloughed-off by-products of the protoplasmic surface. They can be experimentally

removed without sacrificing the life of the protoplasmic body. In plant cells, the extraneous coats of the animal cell are represented by the so-called middle *lamellae*, which lie between the cellulose of neighboring cells or cover the surfaces of single cells. The middle *lamella* consists of salts of polysaccharide pectates. In common with the extraneous coats or walls of animal cells, the pectate wall of plant cells also requires calcium for its structural rigidity. The normal sticky consistency of these external coats depends upon a fairly precise balance in the proportional concentrations of Na and of Ca in the medium, the proportion which exists in Ringer's solution. A decrease in the calcium content or an increase in the acidity of the medium tends to soften and to disperse the pectate *lamellae* of the plant cell and the protein-like extraneous coats of the animal cell.

In studies on permeability, the presence of this material is a factor to be considered, especially when one deals with sheets of coherent cells. Calcium in the medium stiffens and tightens up the intercellular cement so that the resulting decrease in porosity of a cellular membrane is to be explained not so much from changes in the cells themselves as from changes in the cement between the walls. A striking example is the porosity of the blood-capillary wall, which is bathed with a fluid consisting of a balanced salt solution containing calcium. The freedom of the calcium ion to form combinations with the cement varies with physiological changes in pH of the medium.[1] Moreover, changes in the tonic state of the individual endothelial cells may cause the cells either to spread and thin out or to draw in and thicken, thereby narrowing or widening the spaces occupied by the cement between them. The consequent variations in the packing or dispersing of the cement appears to be Nature's way of causing the blood-capillary wall to maintain a readily shiftable state of porosity without affecting the permeability of the constituent cells of the endothelium.

Furthermore, when we deal with single cells, the investigations, for example, on the effect of electrolytes on their water permeability, must take into consideration the possibility of the mechanical effect of a weakened or of a stiffened extraneous coat in permitting or preventing the swelling of cells by the water intake. The stiffening action of calcium on the extraneous coats of sea-urchin eggs might be sufficient to explain the findings of Lucké and McCutcheon[2] that calcium salts decrease the permeability of the eggs to water.

It is of great importance that we realize the existence of such extraneous coats. Jacques Loeb's conception regarding the antagonistic action of salts for the preservation of life may be due, in large part, to the preservation of the extraneous coats of cells by a definite proportion of sodium and of calcium in the medium. The proportional concentration of NaCl and of $CaCl_2$ which R. S. Lillie[3] found to be necessary for preserving the "jelly" which surrounds many echinoderm eggs is the same as that required for extraneous coats in general.

In this discussion on the existence of extraneous coats or cell walls, I am not referring only to material which may be adsorbed, such as the proteins existing in body fluids and in blood, but mainly to coatings which arise

from the cell protoplasm as by-products and are sloughed off from it to form a coat in the presence of calcium or to dissipate in its absence.

A significant experiment which shows the difference in the action of Ca on the extraneous coats and on the naked protoplasmic surface is the following: Unfertilized sea-urchin eggs were immersed in a solution of 0.4 M $CaCl_2$, a concentration which is isotonic with sea water. The extraneous coats of these eggs quickly harden and the eggs behave as rigid balls. When a drop of the $CaCl_2$ solution containing the eggs is deposited on a coverslip, the eggs stick tenaciously to the glass surface. The stiffened coat can be broken and torn with microneedles. The application of pressure with a microneedle on the cracked shell will expel the naked protoplasmic body of the egg, much as a pea can be shelled. The extruded, naked egg immediately assumes the shape of a sphere, it is non-sticky, it can be rolled about and can be pinched into several pieces which immediately round up, each spherule retaining the appearance of normal egg cytoplasm. The spheres behave like a droplet of oil with a flowing, liquid surface. The pinching has to be done with the cylindrical side of the glass microneedle, and the performance can be repeated as long as the surface of the protoplasmic body is not scraped with a sharp edge or the pointed tip of the needle. When this is done, the tear opens and the entire body of the sphere is quickly converted into a mass of frothy, coagulated material. The naked egg spheres can be capped with a drop of oil[4] of appropriate interfacial tension. The liquid state of the surface film enveloping these spheres is demonstrated by the ease with which the capped oil can be moved from place to place and be divided into two separate caps on the surface of the sphere. The effect closely resembles that of a drop of paraffin oil lying on the surface of water, where the oil can be shifted in any direction on the surface but cannot be pulled off.

Evidently, when calcium is present in the medium, the extraneous coats are stiffened, while the protoplasmic surface film is not.

When eggs are denuded of their extraneous coats by shaking and washing in a Ca-free medium and then returned to calcium-containing sea water, the phenomenon of oil-capping also occurs, but in this case the oil cannot be moved about. It would appear that extraneous coat material continues to develop in sea water and forms a covering over the surface of the egg in sufficient quantity to prevent the oil cap from being shifted.

The protoplasmic surface film presumably consists of a palisade-like structure of lipo-protein complexes so arranged that the protein part of the complex is directed to the exterior where it is in direct contact with the surrounding sea water. In sea water, the protein portion of the complex may continually be sloughing off and be denatured to constitute the extraneous coat. It would seem that this does not occur appreciably when the surrounding medium is purely $CaCl_2$.

The mobile protoplasmic surface film can be retained in the total absence of calcium in the medium, such as in an isotonic solution of citrated NaCl and KCl. There is evidence, however, that the sloughing off of protein-like material continues to occur in such a solution but that, in the absence

of calcium, the material becomes dispersed so that no appreciable extraneous coat develops.

We regard the protoplasmic surface film or plasma membrane as a structure which is constantly forming and being renewed. This structure, as long as it is a continuous envelope, maintains the integrity of the protoplasm within and the cell remains alive. The protoplasmic surface film cannot be removed or torn without causing damage. A quick repair of the film may result in recovery. Otherwise, the damage becomes irreparable, whereupon the remainder of protoplasmic surface film dissipates and the exposed interior undergoes disintegration.

We now come to the problem of what is entailed in cytolysis. A protoplasmic body is highly plastic. It can be greatly compressed and distorted. It can be churned[4] to the degree that all parts, including its surface, are displaced without undergoing cytolysis. It is only when the surface film is torn open that cytolysis ensues. The cytolysis can be localized only if the gap of the tear is quickly repaired by the rapid formation of a protoplasmic film beneath the cytolyzed region. In media containing calcium, the cytolytic effect is a conversion of the protoplasm into a dead, sticky, and often frothy coagulum. In the absence of calcium, this does not occur. Instead, the gap produced by the tear opens up, whereupon the granular contents pour out and become dissipated in the calcium-free medium. Under these conditions, the question arises to what extent the integrated constituents of the protoplasm persist so that the granules which are seen to scatter constitute the vital constituents of the protoplasm, or whether other granules appear as products of the cytolytic breakdown. Much work is needed to elucidate this problem.

I wish, here, to refer briefly to the physical state of the protoplasm beneath its surface film. There seems to be no question about the existence of a firm, gelated cortex of appreciable thickness immediately underlying the more mobile, fluid, protoplasmic surface film. Under certain circumstances, the film can be elevated off the cortex. An example of such an occurrence is the formation of the delicate cone-like protrusions on an egg at the site of sperm entry.[4]

The gelated state may occupy the entire interior of the cell. Even in such highly fluid eggs as the *Arbacia* ovum there is evidence that the interior is not a true sol, but a weak, tenuous gel. Apparently, spontaneous variations occur, so that considerable precaution is required in viscosity studies as tested by centrifugation or by the exhibition of Brownian movement.

A similar conclusion, that the cytoplasm of the ameba is normally in a state of incipient gelation, was suggested by Harvey and Marsland[5] in their studies on the ameba viewed through the centrifuge microscope. They noted that the larger crystals in the cytoplasm fell in "jerks" as they were driven by the centrifugal force through a visibly clear field. Moreover, this may have been due not so much to sudden breaks in a colloidal mesh in the fluid cytoplasm, but more likely to occasional sticking of the crystals to the inner border of the peripheral plasma gel. A worth-while check might be to examine the behavior of the crystals in an ameba, the ecto-

plasmic gel of which has been experimentally solated prior to its being exposed to the action of the centrifuge microscope.

In any event, one of the most obvious properties of protoplasm is that of reversible gelation and solation. A gel, no matter how tenuous, offers a consistent structure with plenty of surfaces for the manifold adsorptive and enzymic processes on which protoplasmic function depends.

Concerning the problem regarding the ultrastructure of protoplasm, I wish to refer to the very ingenious conclusion drawn some years ago by A. R. Moore.[6] It is well known that the ameba-like plasmodia of *Myxomycetes* creep through fine pored meshes and reconstitute themselves by fusing together on the other side of the mesh. Moore found that the plasmodia could also crawl through large molecule-sized porcelain filters. However, he observed that viable plasmodial masses appeared on the other side of the filter only when the plasmodium was allowed to crawl through of its own momentum. If he exerted pressure to drive it through, there resulted a breakdown of the protoplasm and cytolysis. He interpreted his experiment to mean that the protoplasm consists of long, fiber-like molecules which, when allowed to orient themselves properly, passed readily through the pores of the filter. However, when extraneous force is applied, many of the fibers would be presented crosswise to the pores and break instead of slipping through. He assumed that this initiated the cytolysis. Here again, there is need of caution. Undue pressure or crushing breaks the protoplasmic surface film which envelopes the creeping plasmodium. This alone, by exposing the interior to the environment, may suffice to induce cytolysis.

The relative inertness of the proteins in the living cell is an interesting feature. This has been suggested by the consistency with which solutions of color indicators of overlapping pH values, when injected into living cells, give color virages pointing to the same pH value of approximately 6.8.[7] It would appear that the proteins present are not sufficiently active to introduce any appreciable protein error such as obtains in the test tube. A similar conclusion based on more positive evidence has been arrived at by Dr. Kopac, which he discusses in a subsequent paper in this monograph.

The fact that there is sufficient ionic activity in the continuous aqueous moiety of protoplasm is indicated by the existence of an appreciable buffering capacity of the protoplasm. A feature of this is the ability of an ameba to tolerate the injection of picric acid. The tolerated amount could be determined by injecting a mixture of picric acid and brom cresol purple. If the injected fluid retains its yellow color, the acidity of the fluid has overcome the protoplasmic buffers and the ameba quickly succumbs. On the other hand, a change of the color to green indicates that the brom cresol purple has assumed the blue color of its alkaline range by the ability of the protoplasm to buffer the introduced acid. These green-colored amebae survive and move about apparently quite unaffected.

The existence of a physiological relationship between the nucleus and cytoplasm is generally accepted. Ocular evidence of such a relationship has been accumulating in the literature, such as the recovery of a fibroblast

from the destructive effect of puncturing the nucleus provided a second nucleus is present in the cell[8] and the experiments which indicate a high degree of porosity of the nuclear membrane of the ameba.[9] Further evidence is presented by Dr. Duryee in this monograph. I wish to emphasize the point that there are certain periods in the life history of a cell during which the interrelationship is carried to the extreme of an actual commingling of the nucleoplasm with the cytoplasm. This occurs prior to proliferative activity of the cell, as in mitosis and strikingly during maturation of an ovum. The commingling of the nuclear sap of the germinal vesicle with the cytoplasm results in the conversion of the cytoplasm into nucleocytoplasm, every part of which is now capable of being induced to undergo cleavage. It is this faculty which in all probability enables a mature ovum to undergo repeated cleavages over a relatively short period of time.

I conclude with a description which indicates the close similarity between plants and animals in their method of cell division. You are aware that the conventional description for an animal cell is that it divides by constriction at the equator, while the plant cell divides by the deposition of a wall across the equator with no evidence of constriction. Actually, we should regard the separation of the two daughter cells as the end stage and only incidental to an earlier and more fundamental process of separation of the protoplasmic bodies of the cell. In this we come back to my thesis at the beginning of my remarks that we must distinguish between the cell and the enclosed protoplasmic body.

Let us consider an animal cell, either the spermatocyte of a grasshopper[10] or the ovum of a sea urchin. The metaphase of the mitotic spindle forms; then the separation of the chromosomes occurs as the anaphase advances into the telophase and the two daughter nuclei begin to be reconstituted. During this period, the animal cell undergoes a certain degree of lengthening (karyokinetic lengthening, first described by Oscar Hertwig), but still with no sign of constriction at the equator. Now a phenomenon occurs which is well recognized in plant but not in animal cells. The so-called telophasic spindle-remnant, at the equator, where the chromosomes had been during metaphase, spreads equatorially, pushing ahead of it all cytoplasmic components which lie in its way until the spreading margin of the spindle-remnant reaches the equatorial periphery of the cell. The separation is now complete between the protoplasmic bodies of the two incipient daughter cells. Not until this is completed do we have the onset of the final phase, which in the plant cells is the deposition of an intervening wall, and in the animal cell an inward advance of the walls of the division furrow.

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SURFACE PROPERTIES OF THE ERYTHROCYTE

By M. H. Jacobs

Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania

The surface of the mammalian erythrocyte has been more studied, by a greater variety of methods, and on the whole with greater success than that of any other cell. There are two chief reasons why it has received so much attention. The first is that it can be physically separated from the cell contents in a manner that is unique among cells. The second is that suspensions of erythrocytes have certain optical properties that are peculiarly favorable for exact experimental work.

Both peculiarities of the erythrocyte are connected with its high degree of specialization for gas transport in the body. In it, internal structures have been reduced to a minimum, and one internal constituent, hemoglobin, has been increased to a maximum. In hemolysis, the hemoglobin escapes from the cell, and little is left but a ghost-like and doubtless somewhat modified cell surface. Erythrocyte "ghosts," suitably purified and concentrated, have been extensively studied by chemical, optical, electrical, and immunological methods.

Furthermore, when the erythrocyte loses its hemoglobin, it becomes almost completely transparent. This loss can be brought about by various alterations of its surface, and special optical methods which are both simple and accurate are, therefore, available for the investigation of surface changes that lead to hemolysis. Similar methods can also be used to study the non-hemolytic volume changes associated with the passage of solutes in either direction through the cell surface. Not the least of the advantages of the erythrocyte for such studies is the fact that the results obtained with it are usually statistical beyond the wildest dreams of the biometrician. Only rarely does an experiment on the erythrocyte involve the use of less than a quarter of a billion of individual cells, *i.e.*, the number contained in one drop of human blood.

By the isolation and study of erythrocyte ghosts, important information has been obtained about the general characteristics of its surface. Since I have done little work of my own in this field, I shall merely summarize very briefly some of the more important conclusions arrived at by other workers.

The erythrocyte ghost has the characteristics of a non-liquid membrane, of the order of perhaps 100 Å,[1,2] or a little more, in thickness. It contains most of the cell lipids,[3] approximately sufficient in quantity to form a bimolecular layer,[4] and large amounts of protein of a rather peculiar nature,[5,6] together with blood-group substances[7] and other chemical materials. It has a high electrical resistance and a large electrical capacity.[8] Dr. Waugh, in the following paper, will discuss its very interesting optical properties. For our present purposes, it is sufficient to note that according to Schmitt and his co-workers[9,10] it behaves as if it contained radially arranged lipid-like molecules and tangentially arranged protein elements.

Various hypothetical models have been proposed to account for these and other known properties of the erythrocyte surface. The simplest of these may be chosen as a basis for discussion, with no present expression of opinion as to its adequacy. In it, the non-polar ends of two layers of radially arranged lipid molecules adjoin each other. The opposite, more polar ends adjoin and may even be in chemical combination with an inner and an outer layer of proteins. Such a model, according to Danielli,[11] while metastable rather than stable, is at least much less unstable than several other conceivable arrangements of protein and lipid molecules.

My own views as to the nature of the surface of the erythrocyte have been derived chiefly from studies of its permeability to various solutes. The remainder of the space at my disposal will be devoted to some of the inferences that can be drawn from such studies. Six principles will be mentioned which seem to govern the behavior of the erythrocyte toward dissolved substances. Any satisfactory hypothetical picture of its surface at best should explain all these principles and at worst should not be incompatible with any of them.

Principle 1. Non-Specific Solubility

About fifty years ago, Overton[12, 13] formulated the principle that the ease of entrance of many substances into living cells is closely paralleled by their solubility in fats and fat solvents or, more accurately, by their partition coefficients between these substances and water. He therefore postulated the presence at the surface of the cell of a layer of material having the general physical properties of a fat-like substance.

Somewhat differently expressed, Overton's principle seems to mean that non-polar hydrocarbon groupings in organic molecules have a general affinity for solvents in which a non-polar hydrocarbon structure predominates. Other things being equal, the higher this affinity in the case of a given solute the greater its partition coefficient between an organic solvent and water, and the more rapid, as a rule, its entrance into living cells, regardless of its molecular volume. On the other hand, polar groups with an affinity for water have the opposite effect, with respect both to partition and to cell permeability. The latter effect is seen with especial clearness in the ionization of many weak organic acids and bases.

Erythrocytes, like nearly all other cells, show many instances of this non-specific solvent-like behavior. Beef erythrocytes, for example, are among the least permeable mammalian erythrocytes to glycerol (molecular weight, 92). Something like three quarters of an hour is required for enough glycerol to enter them from a solution isosmotic with blood to bring about their osmotic hemolysis. But the acetic acid ester of glycerol, monoacetin (molecular weight, 135), despite its higher molecular volume, enters them to the same extent in perhaps 40 seconds. The still larger but more lipid-soluble molecule of diacetin (molecular weight 178) does so in possibly 6 seconds.

The same principle is illustrated by a homologous series of, for example, saturated fatty acids.[14, 15] In the series, acetic, propionic, butyric, and

valeric acids, which differ only slightly in their strengths as acids and are, therefore, particularly favorable for such comparisons, the rate of entrance into the erythrocyte increases with increasing lipid solubility despite increasing molecular weight, in the manner demanded by the principle of Overton. Many other similar examples could be given.

A further likeness in the behavior of the erythrocyte, of many other cells, and of non-specific organic solvents is found in the effect of pH on the uptake of organic acids and bases from an aqueous solution. In general, the uptake of a weak base such as an alkaloid by either an erythrocyte or an organic solvent is favored by alkalinity and hindered by acidity. That of a relatively weak organic acid such as salicylic acid is favored by acidity and hindered by alkalinity.

It seems difficult to account for this universal non-specific type of entrance of even very large molecules into cells without postulating some rather extensive portion of the cell surface in which they can, so to speak, dissolve. Most of the cell lipids are present in the isolated erythrocyte ghost so that a bimolecular layer of them would obviously serve very admirably as a non-specific solvent, though this particular arrangement is by no means the only possible one.

As for the proteins of the cell surface, their hypothetical locus must clearly not be such as to interfere with the free passage of even very large lipid-soluble molecules. An arrangement of the lipids and proteins in parallel, as in a mosaic, would, of course, meet the needs of the situation. An arrangement in series, however, would also be compatible with the observed behavior of these particular molecules if the groupings of the proteins were sufficiently loose to give ready access to the underlying lipid layer. As a matter of fact, there is evidence of another sort that, although proteins are probably present at the surface of the erythrocyte, they by no means form a barrier to non-polar molecules. This is the observation of Mudd and Mudd[16] that erythrocytes introduced into an oil-water interface, unlike leucocytes, go into the oil. The presence of additional substances at the surfaces of sensitized erythrocytes, however, may destroy their affinity for oil.[17]

Principle 2. The Molecular Sieve Mechanism

The erythrocyte, like other cells and certain artificial membranes, is, in general, more permeable to very small molecules than to those of larger size. Indeed, molecules which are sufficiently small seem to enter it with great ease, however low their affinity for lipids.

As a molecular sieve, the surface of the erythrocyte behaves as if it were either very thin or very porous, or both. Its permeability to the most abundant of all small molecules, water, is greater than that of any other known cell. Lucké[18] has tabulated comparable permeability constants for a considerable number of cells of both plant and animal origin. Expressed in the same units, they range from about 0.1 to 1.0. The corresponding figure for the erythrocyte is about 3.0,[19] that is, 3 to 30 times as great. Even this figure is probably too low, since it depends upon a

hemolysis method, and the escape of hemoglobin from the cell is by no means instantaneous. For a time, the endothelial cells of the capillary wall[20] were thought to be even more permeable to water than the erythrocyte, but, according to Zweifach,[21] it is the permeability of the intercellular substance rather than that of the cells themselves that is here involved.

The permeability of the erythrocyte to small hydrophilic molecules varies so greatly from species to species that generalizations are difficult. At the one extreme, beef erythrocytes[22] show a slightly lower permeability to ethylene glycol and glycerol than do cells of the plant, *Chara*,[23] and *Arbacia* eggs.[24] At the other extreme, the erythrocytes of some rodents, particularly the groundhog,[25] show evidence of a porosity greater than that of any other known animal cell (provided that the observations of Zweifach, mentioned previously, permit the exclusion of the cells of the capillary endothelium).

Further light on this type of permeability seems to be thrown by a study of the effect on it of different temperatures. Temperature coefficients (Q_{10}) for the rates of entrance of different hydrophilic solutes vary widely, *i.e.*, from about 1.4, which is typical of a simple diffusion process, to 6.0 or more, but with a very general tendency of the values, with the same cell, to increase with the molecular weight of the penetrating substance.[26] High temperature coefficients are frequently supposed to indicate that some chemical reaction is involved, but there is no independent evidence to suggest that this is true of the entrance of most solutes into the erythrocyte. Even if it were, there is no obvious reason why the thermal characteristics of the reactions in question should be so directly related to the sizes of the diffusing molecules. Much more plausible is the suggestion of Danielli and Davson[27] that the diffusing molecules must force their way through a barrier of some sort, and that only those individual molecules succeed in doing so whose kinetic energy reaches a certain critical value.

What part of the cell surface is capable of acting as a molecular sieve? Collander[28] has studied artificial protein membranes and found them to have sieve-like properties. But a uniform protein layer at the surface of the erythrocyte, in series with the lipids, could hardly act in this way. If its meshes were large enough to provide a ready passage for large lipophilic molecules, it could hardly exercise a selective action according to size on much smaller hydrophilic ones. It would seem that an effective sieve-like protein structure would have to be arranged in parallel with the lipids—which is essentially the old mosaic theory of cell permeability. It is entirely possible, however, that the lipids themselves might act as the molecular sieve. Rideal[29] and Langmuir[30] have shown that a monolayer of a fatty acid permits a fairly ready passage of water molecules through it. The more complex phospholipids of the erythrocyte might easily provide a still greater degree of porosity.

Principle 3. Species Differences in Permeability

In their permeability to hydrophilic solutes, specific differences among erythrocytes are both striking and numerous. First, as has just been mentioned, there are differences in the apparent pore size of the molecular

sieve. The erythrocytes of the beef and sheep admit the 3-carbon glycerol molecule with some difficulty. Those of the groundhog are entered readily by 4-, 5-, and even 6-carbon compounds.

Of greater interest are cases in which differences in chemical structure rather than in mere molecular size are involved. For example, human erythrocytes are very slightly permeable to the 4-carbon polyhydric alcohol, erythritol, but they are entered with considerable ease by the 5-carbon sugar, xylose, and they even have a permeability, not found in most other species, to the 6-carbon sugar, glucose. In the mouse, conditions are the exact reverse. Its erythrocytes show little permeability to any of the sugars, but they are unusually permeable to the 4- and 6-carbon polyhydric alcohols, erythritol and mannitol.

The molecules of mannitol and glucose are so large that any pores in a simple molecular sieve which would admit them should also admit the smaller molecules of erythritol and xylose. A door that the cat could enter would not keep out the kittens. Some factor other than molecular volume or lipid solubility—the latter being very low for all the substances mentioned—seems to be operating in such cases.

Specificity is not confined to large molecules, but extends to those of small size as well. Urea and ethylene glycol both have low and approximately equal molecular volumes. But, whereas most cells, including the erythrocytes of all the lower vertebrates, are more permeable to ethylene glycol and sometimes even to glycerol than to urea, mammalian erythrocytes are incomparably more permeable, *i.e.*, 100 times or more, to urea than to ethylene glycol, or indeed to most other substances.

Glycerol penetrates some erythrocytes (*e.g.*, those of man and the rat) at a rate that is much faster than would be predicted from its molecular volume and low lipid solubility. It also seems to enter the erythrocytes of most of the birds that have so far been studied as fast as, or sometimes even faster than its smaller homologue, ethylene glycol. The erythrocytes of the reptiles, on the other hand, show a very low permeability to glycerol.

Among the mammals, the erythrocytes of almost all the carnivores so far studied show a peculiar sensitivity to the action of alkalinity that is not found in other groups. Even very closely related species of mammals may show characteristic differences in permeability. We have found at least a dozen apparently constant differences in permeability between the erythrocytes of the albino rat and the albino mouse.[31] For my own amusement, rather than for any useful purpose, I have constructed an analytical key, based on permeability characters and similar to those used by systematic zoologists and botanists, which permits the identification of the erythrocytes of some 20 species of mammals.

This remarkable specificity of the cell surface has not been observed to apply to lipid-soluble substances, which show a great similarity of behavior with all erythrocytes and with most other plant and animal cells as well. In the case of the highly varied behavior of the hydrophilic substances, however, it seems impossible to account for the known facts, of which only a few have been mentioned, without introducing into our hypothetical

picture of the cell surface a rather high degree of chemical complexity, involving the presence of substances other than lipids and proteins. What the nature of these substances may be, and how they are arranged in the cell surface, is still a matter for speculation. But increasing knowledge is not likely to simplify any picture that has so far been suggested.

Principle 4. Special Permeability to Anions

A special permeability to anions seems to be peculiar to the erythrocyte and is known to play an important part in the transport of carbon dioxide by the blood. This permeability is sufficiently great to permit large exchanges of Cl^- and HCO_3^- ions during the three quarters of a second or less which the blood spends in passing through the capillaries of the lungs, with a reversal of the exchange during its passage through the body tissues. A similar rapidity of exchange has been shown by several methods to occur *in vitro*, and is known to be possible in the case of many other relatively small anions such as OH^-, Br^-, NO_3^-, NO_2^-, *etc.* The erythrocyte, however, is practically impermeable to large anions such as ferricyanide, citrate, tartrate, *etc.*

With regard to cations, the situation is somewhat complex. It is certain that a free permeability to them cannot exist. If it did, the erythrocyte could not maintain its integrity, since it is easy to show that simultaneous osmotic and ionic equilibria could not exist at a finite cell volume. On the other hand, there are observations which indicate that the impermeability of the erythrocyte to cations is neither purely physical in nature nor entirely complete. According to Harris,[32] metabolic processes in the erythrocyte are involved in the retention by it of potassium. Its impermeability to this ion therefore, at least in part, may be a physiological one. Experiments with radioactive potassium and sodium[33, 34, 35] also show that slow passages of these ions across the erythrocyte surface can occur, but that the times required are measured in hours rather than in fractions of a second, as is the case with anions. Practically speaking, therefore, the older concept of the erythrocyte as a cell freely permeable to anions and impermeable to cations is still valid for the interpretation of brief experiments, though more complex metabolic factors must be taken into account when the times are long.

There is good reason to believe that the differential permeability of the erythrocyte to ions is associated with the protein portions of its surface. One piece of evidence is the fact that this differential permeability is destroyed by a variety of agents that denature proteins. It has been suggested, from time to time in the past, that many kinds of hemolysis may fundamentally be due to a conversion of a cation impermeability into a cation permeability by the hemolytic agent. This view has recently been elaborated and given experimental support by Wilbrandt,[36] who has proposed the term "colloid-osmotic hemolysis" for the indirect osmotic hemolysis resulting from cation permeability.

One agent which produces changes of this sort in an easily controllable way is butyl alcohol[37] in concentrations approaching saturation of an aqueous

solution. The effect falls off rapidly with decreasing concentrations. It is very easy to demonstrate the production of cation permeability with butyl alcohol. The procedure is to expose the cells for an appropriate time to a high concentration of the alcohol in an isotonic solution of sodium chloride or of sucrose and, then, before hemolysis has occurred, to dilute the mixture with a considerable excess of isotonic sucrose, which reduces the concentration of the alcohol to a point at which it has little further effect on the cells. If the exposure of the cells is too long, they become permeable to sucrose as well as to sodium. If it has been properly chosen, however, a suspension is obtained of erythrocytes which are stable in sucrose, but which almost instantly undergo hemolysis when placed in a salt solution. Their volume changes, in different mixtures of sucrose and salts and in salt solutions of different pH values, are in good agreement with the theoretical predictions for cells permeable to both anions and cations. Other methods of denaturing the proteins may also be used to obtain cation-permeable cells—for example, by heating the cells, either with or without the addition of a substance which favors protein denaturation, such as salicylate or urea, and then quickly cooling them in a solution consisting chiefly of isotonic sucrose.

What is the location in the cell surface of the proteins responsible for differential ionic permeability? If the arrangement were in series with the lipids, it is difficult to imagine that any superficial wide-meshed layer of protein through which large lipid-soluble molecules could readily pass would delay for hours the passage of, for example, radioactive potassium ions. Apart from this difficulty, however, would be that of accounting for the passage of hydrophilic ions through the strongly hydrophobic underlying lipid layer. Indeed, the high electric capacity of cell suspensions, including those of erythrocytes, is generally interpreted as indicating extensive ion-impermeable regions, which are presumably furnished by lipids.

It seems plausible, therefore, to suppose that any protein regions which show differential permeability to ions are in parallel with the lipids rather than in series with them. This is essentially the conclusion arrived at by Cole and Curtis as a result of electrical studies on cells of a very different nature. With the giant axone of the squid[38] and with *Nitella*[39] cells, they found on stimulation an enormous increase in electrical conductance, indicating increased ionic permeability, but associated with only a minimal decrease in the electrical capacity, which depends on ionic impermeability. Cole[40] has plausibly interpreted these findings as indicating that permeability to ions is confined to a relatively small part of the cell surface.

Principle 5. Differential Changes in Permeability

Space will permit the mention of only two examples of this principle. Several years ago, my assistant, Mr. Corson, happened to make up a glycerol solution in some commercial distilled water and, on using it with human erythrocytes, obtained a surprisingly low rate of penetration of the solute. We were later able to determine that this effect was due to traces of copper in the water and that it could be imitated by adding a copper salt to ordinary

distilled water in concentrations of 10^{-5} M and lower. Further work has shown that under favorable conditions it can be obtained at concentrations down to the order of magnitude of 10^{-8} M, and that it occurs with the erythrocytes of only a few of the many species of mammals so far investigated—of which those of man show it most clearly. It is also specific for glycerol. The only one of several dozen other substances studied that behaves at all similarly is monoacetin, which is a glycerol ester. The only other metal that closely resembles copper is mercury, but even it behaves differently in several important respects. Under favorable conditions, copper may decrease the permeability of human erythrocytes to glycerol to only 10 per cent of its original value, while leaving that to other hydrophilic non-electrolytes, to lipid-soluble substances, and even to anions essentially unchanged.

Not long after the discovery of this effect on the erythrocyte, Langmuir[41] reported a striking effect of copper in similarly low concentrations on monolayers of fatty acids. We were therefore tempted for a time to look to the lipids of the cell for an explanation of our effect. But we gave up the attempt because of two chief considerations. The first is that it is difficult to see how copper could change the physical properties of the cell lipids to the extent of almost abolishing glycerol permeability, without at the same time producing some parallel effect on their permeability to many other substances. The second is that all erythrocytes, and probably all other cells, seem to have lipid elements in their surfaces, but in only a few species is their permeability to glycerol strongly affected by copper. These, it turns out, are all species into whose erythrocytes glycerol enters faster than it should, *i.e.*, faster than would be predicted from its molecular volume and its low solubility in lipids. The copper effect seems to be very strong in the erythrocytes of the birds, mentioned above, into which glycerol enters as rapidly as ethylene glycol. Another reason for believing that the lipids are not primarily concerned in the copper effect will be discussed under principle 6 below.

By way of contrast with the specific effect of low concentrations of copper in decreasing the permeability of human erythrocytes to glycerol and leaving it almost unchanged to other non-electrolytes and anions, is that of tannic acid. This substance in low concentration strongly decreases the permeability to anions of the erythrocytes of several species so far studied, without at the same time, at body pH, much affecting their permeability to glycerol and other non-electrolytes. Tentatively, it seems plausible to suppose that the tannic acid acts upon the same protein regions whose permeability to ions is so profoundly altered by denaturation. The characteristic action of tannic acid on proteins is equally well shown in the tanning of leather and in its effects on protein monolayers.[42]

Space will not permit a discussion of the effect of narcotics on the permeability of the erythrocyte. It can only be said that this effect is highly complex. In low, non-injurious concentrations these substances seem typically to decrease the permeability of all erythrocytes to anions, to increase that of all erythrocytes to at least some lipid-soluble substances, but, in the

case of glycerol, to decrease it markedly in some species and not at all in others. Such a complexity of effects would appear to demand for its explanation an equal complexity of structure.

Principle 6. Spatially Limited Permeability

The effect of copper on the permeability of the human erythrocyte to glycerol has been mentioned. A quantitative study of it has yielded some suggestive results which must be incorporated in our hypothetical picture of the molecular structure of the surface of the erythrocyte. In a typical experiment, when approximately 250 million cells were exposed to 20 ml. of a glycerol solution containing a concentration of $CuCl_2$ of 6×10^{-7} M, their permeability to glycerol was reduced to about 10 per cent of its original value. It is easy to calculate that, if in producing this effect all the copper atoms in the solution were taken up by the cells, there would be 30,000,000 of them for each cell. Other experiments have shown in several ways that the effect of the copper is produced at the cell surface rather than in its interior, and that it is almost instantaneous and completely reversible. From the known dimensions of the copper atom and of the human erythrocyte, it readily follows that, in the case just mentioned, the copper atoms could cover at most only a little more than 1 per cent of the total cell surface, despite the fact that they lower its permeability to glycerol by 90 per cent of its original value.

But this estimate is certainly too high. If erythrocytes are exposed to such a dilute copper solution and then quickly removed from it by centrifugalization, it can be shown that most of the copper remains in the solution rather than attached to the cells. In one such experiment, it was estimated that each cell could have taken up no more than 1,000,000 copper atoms, and these would suffice to cover no more than 1/2000 of its total surface. Figures such as these suggest that any effect of the copper on a number of fatty-acid molecules sufficient to form a bimolecular layer could hardly be expected to cause any very profound change in the physical behavior of such a layer.

In view of the highly specific nature of the copper effect for glycerol, in view of the fact that it appears only in erythrocytes which are "abnormally" permeable to glycerol, and in view of the further fact that spatially it appears to be confined to a very small part of the cell surface, it seems reasonable to suppose that copper produces its characteristic effect on the human erythrocyte by acting on some localized mechanism for glycerol transport not found in most erythrocytes. In favor of this view is the fact that, when maximally inhibited by copper, human erythrocytes still retain a rather low permeability for glycerol of about the same magnitude as that found in most other species of mammals, in which a molecular sieve mechanism presumably operates It is tempting, therefore, to think that the special mechanism in question in the highly permeable cells is an enzyme. Support for this view is furnished by a recent short note by LeFevre,[43] who reports a similar specific inhibition of glycerol uptake by p-chloromercuribenzoate and other substances which, like copper, have an inhibiting effect on a number

of known enzymes containing -SH groups. Further details concerning this work will be awaited with interest, but in the meantime it should be noted that there is nothing improbable, or even new, about the idea that enzymes may be present in the cell surface. Tentatively, therefore, a place should be made for them in our hypothetical model.

The chief conclusion to be drawn from these studies can be summed up in a single word: "complexity." Simple models, such as that originally suggested, have their usefulness as a starting point for theoretical discussions. When they fail to account for all the known facts, however, they must be modified. If in the process they lose their simplicity, we need hardly feel surprised. Protoplasm in general is universally believed to have a very complex structure. Why should its strategically placed and enormously important surface be less complex in structure than its other portions?

A good deal has been made in the past of the fact that a complex mosaic type of structure of some sort, to which the known phenomena of cell permeability at present seem to point, is physically less stable than the model chosen for discussion. This difficulty does not seem to me to be a very serious one as far as the erythrocyte is concerned. In the first place, the erythrocyte surface, whether as a ghost or as a part of an intact cell, does not seem to have the properties of a simple liquid. There is some evidence that even its lipids may, at least in part, be in chemical combination with underlying proteins, which in turn may form a part of a continuous framework, or stroma. In the second place, whether or not stability is secured in this way, the fact of instability in itself presents no theoretical difficulties. Protoplasm is a notably unstable system, maintained only by a constant expenditure of energy. There is no logical reason why its surface should have fundamentally different properties.

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THE ULTRASTRUCTURE OF THE ENVELOPE OF MAMMALIAN ERYTHROCYTES

By David F. Waugh

Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.

One can support the suggestion that most cells maintain a thin layer of surface material distinct from the underlying cytoplasm. This layer is to be distinguished from materials, such as cellular and cementing substance, which are considered as cellular secretion products. This thin layer, or plasma membrane, may be demonstrated by many techniques, such as the microdissection techniques of Chambers, optically by polarized light technique, and in other ways. With polarized light technique, the limiting material is distinguished, since it is doubly refracting and shows, in many cells, a distinct polarization cross. This method not only reveals molecular orientation in the plasma membrane but also in the nuclear membrane and in many other cellular components, such as chromosomes, the spindle and asters, mitochondria, *etc.*[1]

For those cells in which materials must pass through in a molecular state, it seems reasonable to suppose that the plasma membrane is the agency which allows differential passage or differential permeability. The eventual determination of the ultrastructure of the various plasma membranes should give a solution to this problem. In some cells, such as the proximal tubule cell of the kidney, a distinct limiting membrane is not demonstrable. In these cases the molecular structure of the plasma membrane may have little to do directly with "permeability," the entrance of materials into the cell being controlled in other ways, possibly by vacuole formation.

Although the techniques mentioned may demonstrate the presence of an outer layer having distinct molecular orientation, the presence of cytoplasm in close association has, in most cells, so far prevented a structural analysis. The mammalian erythrocyte presents an interesting exception. In these cells, as indicated by Jorpes,[2] and now generally agreed upon, the non-hemoglobin protein is present largely in the envelope. In addition, Erickson *et al.*[3] have found by chemical analysis that the total amount of lipid is also present in the envelope. This suggests that the internal cytoplasm of the red cell is small compared to the envelope material. This view is strengthened by the investigations of Fricke, Parker, and Ponder,[4] which indicate that the internal framework would not require more than about 0.1 per cent stromatin, a negligible amount compared to the envelope. Hemolysis serves to remove hemoglobin and salts. Where this can be done effectively, the envelope remains intact and may be studied by a variety of methods.

It seems obvious that the erythrocyte envelope may not be a typical "plasma membrane." In this respect, a complete analysis in which structural, permeability, and metabolic properties are correlated may not offer a general solution applicable to other cells—each may require separate analysis. Jacobs has shown, however, that the erythrocyte presents a challenging array of permeability problems. A solution to these should further our insight into similar situations presented by other cells.

Polarization Optics.[1, 5] A regular (crystalline) array of anisometric molecules, such as a fatty-acid crystal, has different properties in different directions. These include heat transfer, electrical polarization, cleavage, and effect on light. For present purposes, consider a fatty-acid crystal (FIGURE 1). A plane which includes cross sections of the chains (A) has no distinguishing directions, and light which enters the crystal perpendicular to this plane will emerge unchanged. This direction is termed the optic axis. Observation in a plane which includes the fatty-acid chains (B) reveals two distinguishing directions immediately. A beam of polarized light which enters the crystal perpendicular to this plane may or may not be altered as follows: If the plane of vibration of the polarized light is parallel or perpendicular to the fatty-acid chains, it will proceed unchanged. If the plane of polarization makes an angle with the fatty-acid chains, the incident beam will be broken into two components vibrating parallel and perpendicularly to the fatty-acid chains. The two beams thus produced will travel with different velocities in the crystal and may emerge out of phase. Thus the emergent beam is usually elliptically polarized. The difference in velocity of the

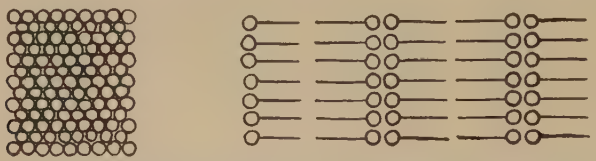


FIGURE 1. Schematic representation of a fatty-acid crystal whose chains are oriented perpendicularly to the planes of the carboxyl group. *Left* (A): a plane cutting at right angles to the hydrocarbon chains. *Right* (B): a plane cutting parallel to the chains.

two beams produced by the crystal means that the crystal has two refractive indices. One of the rays represented by these indices behaves according to Snell's law and is termed the "ordinary ray." The other does not and is termed the "extraordinary ray." The sign of the double refraction is obtained by subtracting the refractive index of the ordinary ray (N_o) from that of the extraordinary ray (N_e).

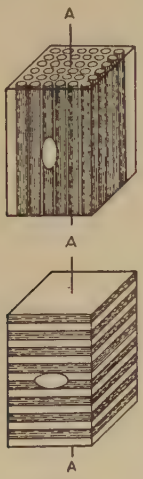
The fatty-acid crystal is a positive uniaxial crystal. The ray whose electric vector vibrates parallel to the fatty-acid chains (the direction of the optic axis in FIGURE 1) is the extraordinary ray, and the refractive index is greatest in this direction. The double refraction described is crystalline double refraction and is exhibited by most lipids and protein systems of oriented polypeptide chains. The optic axis lies parallel to the directions of the main molecular chains in both cases. Since biological materials are uniaxial, a discussion of more complicated types of crystals will be omitted. The presence of double refraction may be tested for by placing the object between two polarizing (Nicol) prisms. The prisms are oriented so that the polarized light produced by the first cannot pass through the second. The field is dark. If the object is oriented so that one looks down the optic axis, the field will remain dark on rotating the object. In directions other than the optic axis, the fatty-acid crystals will appear dark when the long direc-

tions of the chains are oriented parallel or perpendicular to the polarizing directions of the Nicol prisms. In other directions, the crystal will appear bright and will have maximum brightness at 45° orientation.

Double refraction is an intrinsic property which is independent of the size and shape of the system. The phase difference between the extraordinary and ordinary rays, Γ, is given by the product of double refraction and thickness, *d*. Thus:

$$\text{Double refraction} = n_e - n_0 = \frac{\theta\lambda}{d} = \frac{\Gamma}{d},$$

where θ is the phase difference and λ is the wavelength of light used. One ordinarily measures on retardation and divides this by thickness to get



$$n_e^2 - n_o^2 = \frac{\delta_1 \delta_2 (n_1^2 - n_2^2)^2}{(\delta_1 + 1) n_2^2 + \delta_2 n_1^2}$$

$$n_e^2 - n_o^2 = -\frac{\delta_1 \delta_2 (n_1^2 - n_2^2)^2}{\delta_1 n_1^2 + \delta_2 n_2^2}$$

FIGURE 2. Rodlet and platelet form double refraction. The directions A indicate the optic axes. The corresponding Wiener equations are given on the right. Taken from Schmitt, J. Applied Physics **9**: 109, 1938.

double refraction. A variety of compensators are available for determining signs of double refraction and retardation. For objects, such as the erythrocyte envelope, having little retardation, a thin mica plate having a total retardation of $\lambda/20$ is placed in the optical path and used to compensate for the double refraction of the object. This is termed the Köhler compensator and, since its sign is known, the direction of rotation necessary for compensation gives the sign of double refraction of the material.

In addition to crystalline, double refraction systems of oriented rodlets or platelets, whose size is large with respect to the wavelength of light, will also show double refraction called "form" double refraction. O. Wiener, in 1912, calculated the behavior of such systems and derived formulae assuming that the particles have no crystalline double refraction of their own. For rodlets and platelets the situation is shown in FIGURE 2, which also shows the directions of the optic axes.

In these, δ_1 and δ_2 are the partial volumes of the particles and surrounding medium, respectively, and n_1 and n_2 are their refractive indices. Rodlet form double refraction is always positive, while platelet form double refraction is always negative. It is to be noted that form double refraction depends upon the difference between the refractive indices of particles and medium. If they are made equal by suitable media, the form double refraction disappears.

Most cellular materials have components of form double refraction and crystalline (or intrinsic) double refraction. According to the Ambronn method, they may be separated, in instances, by immersing the material in a series of media of different refractive indices. FIGURE 3 indicates the

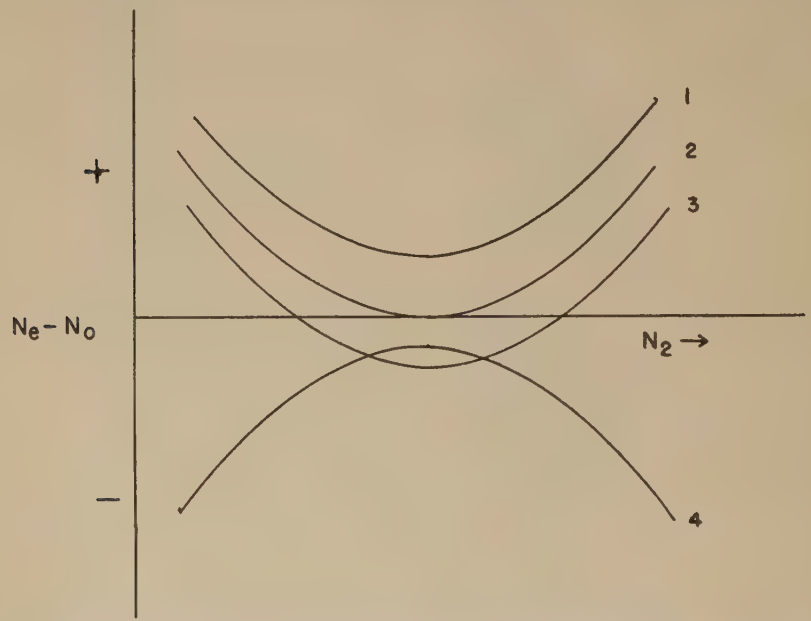


FIGURE 3. Dependence of double refraction on the refractive index of the immersion medium (N_2). Curve 1, rodlet form double refraction and positive crystalline double refraction. Curve 2, rodlet form double refraction only. Curve 3, rodlet form double refraction and negative crystalline double refraction. Curve 4, platelet form double refraction and negative crystalline double refraction.

results where double refraction is plotted as ordinates against the refractive index of the immersion medium. The minimum in the hyperbolic curves occurs when form double refraction has been eliminated ($n_1 = n_2$). This minimum, therefore, measures the refractive index of the particles if no complications occur. If the minimum occurs at 0 ordinate, the system has form double refraction entirely and the particles themselves are isotropic. If it is + or −, the double refraction at the minimum is the magnitude of crystalline double refraction due to the intrinsic structure of the particles. Form double refraction is generally associated with protein rodlets or platelets.

Normal red cells are too opaque to show polarization effects. After hemolysis by freezing and thawing according to Schmitt, Bear, and Ponder,[6]

and suspending in 1 per cent NaCl, the envelopes of rabbit cells show, if anything, a faint negative cross with the optic axis directed radially. After treatment with lipid solvents, the envelope has a definite form double refraction negative cross indicating protein leaflets or platelets tangentially oriented. Immersion in glycerol or urea solutions, which increase the refractive index of the immersion medium and decrease the protein form double refraction, immediately produces a positive cross with radially directed optic axis indicating oriented lipid, the long axes of the lipid molecules also being radially directed. The envelope appears, therefore, as a lipid-protein complex.

Since the thickness is below the resolving power of the microscope, the physical dimensions of the envelope do not permit one to calculate double refractions. Under normal circumstances, however, the form double refraction of the protein balances the intrinsic double refraction of the lipid component. The double refraction data establish with some certainty the orientations of the lipid and protein components in the envelope.

The Electron Microscope. A detailed discussion of the electron microscope is not necessary here. The instrument is familiar to most and the high degree of resolution, 50 Å, is present-day legend.[7] Wolpers[8] has prepared stroma by osmotic hemolysis followed by osmium fixation and estimates a membrane thickness of the order of 250 Å. If such cells are not fixed, the thickness is greater. He estimates the thickness in whole cells to be about 150 Å. Such figures are approximations only.

During the course of removal of lipid from osmotically hemolyzed cells, defects or holes appear in the envelope. If such extracted membranes are fixed with osmic acid, a fibrous structure is evident. The fibrils have widths of about 150 Å after this treatment. They may be considerably smaller *in vivo*, for the osmic acid may exert some condensing effect. Wolpers points out that the fibrous structure appears after the several steps in a rather drastic treatment: osmotic hemolysis, lipid extraction, osmium fixation, and high vacuum drying.

Wolpers views the envelope as being composed of a scaffolding or felt work of protein fibrils in which the lipid molecules are dispersed.

The fibrous nature of the protein component of the envelope was anticipated chemically by the work of Boehm in 1935.[9] Boehm dispersed stroma in lithium perchlorate and found that the resulting dispersion had a high viscosity and showed intense streaming double refraction.

The envelope material seems disposed to the formation of fibrous structures. Furchgott[10] treated suspended ghosts with lyotropic salts such as lithium perchlorate and potassium thiocyanate, much as Boehm had done, and found, using dark field technique, that the envelopes "unravelled" into long thread-like structures, networks of threads, or medusae. This is, apparently, a spontaneous reaction on the part of the ghosts, which can be lost by prolonged washing.

From evidence so far presented, it would appear that the protein leaflets responsible for negative form double refraction are composed of protein fibrils or filaments. Orientation or disorientation of fibrils within the plane of the protein leaflet remains to be demonstrated. Over large areas, there is

probably statistical disorientation.[11] Likewise the lateral extent, or average area, of the leaflets has not been determined. These results are in agreement with double refraction analysis. Double refraction does not specify the internal structure of the protein leaflet component.

Reflectivity Method. A direct measurement of the thickness and refractive index of the dried envelope before and after extraction with lipid solvents is possible with the leptoscope.[12, 13] Blodgett and Langmuir[14] and Blodgett[15] have made a careful analysis of the changes in intensity of light which is reflected from a glass surface covered by a thin transparent film. The variables involved are the refractive indices of film, supporting material, and medium (usually air) through which they are viewed, the angle of incidence and wavelength of the light, and the thickness of the film. By utilizing the

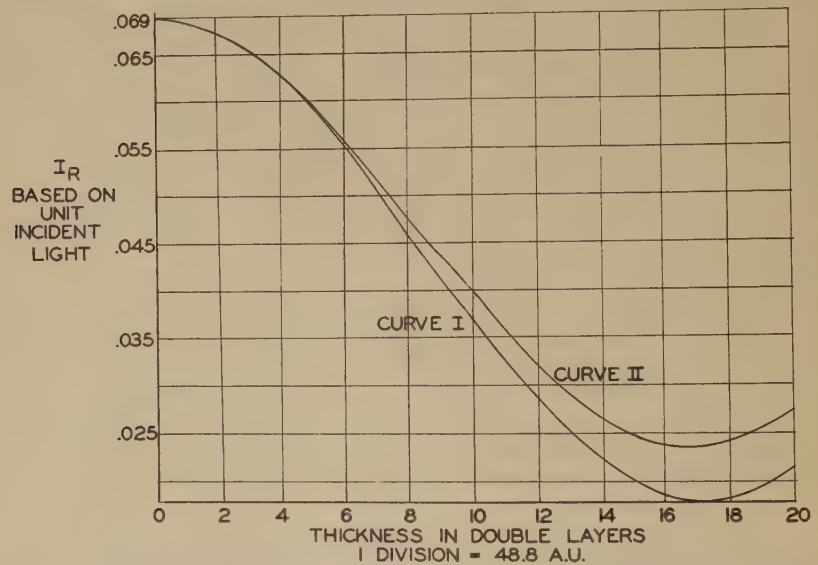


FIGURE 4. Reflectivity of a glass of Ng = 1.70 at λ = 5,000. The curves are based on unit normal incident intensity. Curve I refers to a film of barium stearate having a refractive index of 1.495, while Curve II refers to a film (possibly protein) having a refractive index of 1.525.

film-building technique of Langmuir and Blodgett, in which a plate is dipped through a monolayer of mixed stearate and stearic acid under pressure, one can deposit a film having *steps* of 48.8 A.U. thickness increment. These films have a refractive index of 1.495 and, when viewed at vertical incidence, will conform to Curve I of FIGURE 4. The wavelength of the light is assumed to be 5000 A.U. and the refractive index of the glass 1.70. The ordinates are based on unit incident intensity and the abscissa represents film thickness in double layers. Curve II represents a film having a refractive index 1.525 under the same conditions. One observes that the intensity-thickness curve is a cosine squared curve, that the change in intensity for a given small thickness increment is largest over the central portion of the curve, and that the first minima occur at about 17 double layers or 830 A.U.

The difference of intensity between maxima and minima depends upon the difference in refractive index between film and glass. This should be as large as possible.

By using a set of glass plates covering a range of refractive indices in small increments, one can measure the refractive index of a film by noting that glass on which all thicknesses of film reflect the same intensity of light as the clean glass. This serves as a convenient method for measuring the refractive indices of materials in micro quantities (10^{-10} gm.) or in quantities which, though still small, are large enough to yield a film visible to the eye.

These observations may be applied to biologic membranes by depositing such membranes on a glass of high refractive index and observing them with a suitable technique. The technique used previously, and one which is

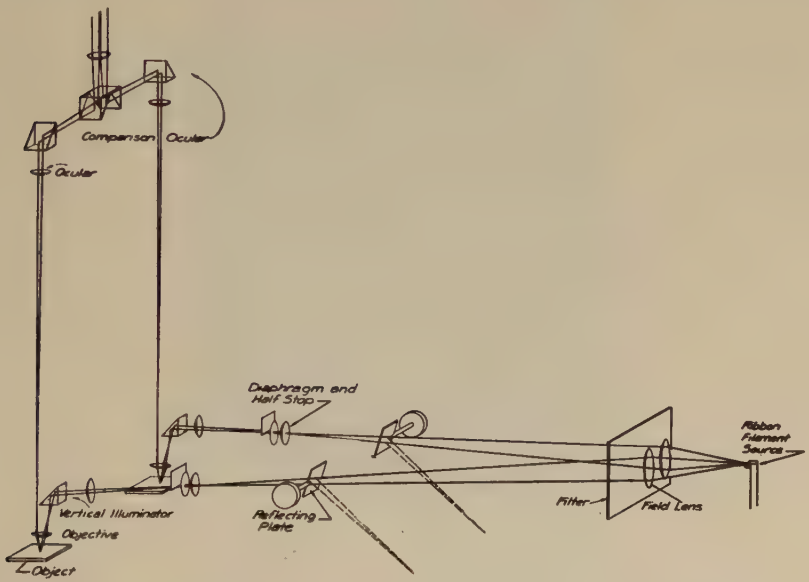


FIGURE 5. Optical system of the leptoscope.

under modification at present, is shown in FIGURE 5. For obvious reasons, the technique utilizes microscopes. Since *reflected* light is to be observed, one resorts to the vertical illuminator, a device in which the light which enters horizontally is reflected downward to the objective. The objective focuses the light on the object which reflects a portion back to the objective. At this time, the objective serves its normal function. It picks up the reflected light and focuses it toward the objective. The system used is a double one having two microscopes and two vertical illuminators with a single source of light. The envelope preparation is made on one slide and is placed on the microscope. The other microscope accommodates a built-up film of barium stearate having a number of steps from 0 on up, each differing by a single double layer of stearate. The comparison ocular views one half of the field from each microscope, thus allowing a direct comparison to

be made between the dried envelopes and a film of known thickness. For details see References 12 and 13.

The envelopes are prepared as follows. While in a horizontal position, a clean slide is covered with a few ml. of a highly dilute suspension of washed red cells. During the next two minutes, some of the cells will settle and adhere to the slide. The excess cells are washed off with isotonic saline; the slide is drained and then immersed in a 10^{-4} M buffer made with double distilled water. The dilute buffer hemolyzes the cells. While the slide is immersed, the surface of the hemolyzing buffer is covered with a monolayer of egg albumin or other protein. After 1 minute, the slide is then dried in a vertical position. Drying takes about 2 minutes. This time is considered as being part of the hemolysis time. The protein monolayer lowers the

FIGURE 6. View through comparison ocular of leptoscope. Double envelope discs are shown on left. On right is a step film showing, above, 6 double layers and, below, 7 double layers.

surface tension of water sufficiently to prevent disruption of the envelopes when they reach the interface. There is sufficient residual tension, however, to draw them into two layered discs and they dry as such, as shown in FIGURE 6. This is a photograph of the field through the ocular of the leptoscope showing a film of 7 and 6 double layers on one side and envelope discs of the rabbit on the other. To measure thickness one moves the built-up film until a matching step or interstep is obtained. The known thickness of this step and the refractive index of the envelope are used to calculate actual envelope thickness. The refractive indices of most unextracted envelopes are sufficiently close to the film refractive index to require no correction.

The slide with the dried envelopes may be dipped in lipid solvents and material thus extracted. A remeasurement of refractive index and thickness now gives a measurement of the material taken out of the envelope.

The dried envelopes have varying diameters. The measured thicknesses thus are usually not the thicknesses of the envelope *in vivo*. Comparable thickness values for different species may be obtained by calculating the volume of material in the envelope disc and dividing this value by the area of the erythrocyte *in vivo*. Values given by Ponder in his monograph of 1934[16] have been used. The values are rabbit $106\mu^2$, rat $106\mu^2$, and steer $63\mu^2$.

The results for rabbit erythrocytes are typical (FIGURE 7). In this, the thickness of the envelope in A.U. is plotted as ordinate with the pH of the hemolyzing buffer as abscissa. The maximum normal thickness occurs at pH 6.0 and is about 215 Å ± 15 Å. On either side of this pH, the thickness decreases rapidly indicating loss of material. These same cells, leached in a mixture of chloroform and alcohol (2:1) brought to boiling for 15 min., gave

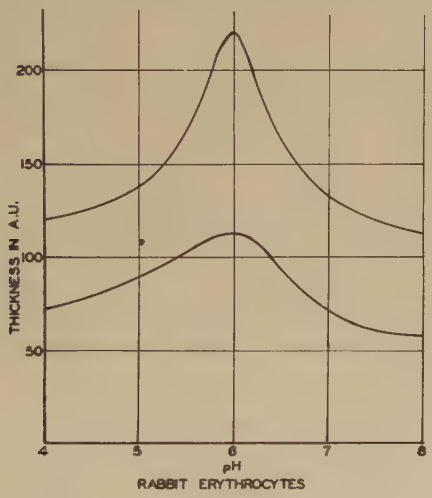


FIGURE 7. Thickness in Ångstrom units of erythrocyte membrane of rabbit cells versus the pH of the hemolyzing buffer.

thicknesses as shown in the lower curve. The composition of the envelope seems relatively constant, indicating that the disruption by hemolysis removes components of constant composition and not protein or lipid soluble materials selectively. The thickness of leached cells is about 110 A.U., giving a volume ratio of lipid to protein of 1 to 1 or a weight ratio of 0.7 (assuming lipid density of 0.9 and protein density of 1.3).

Much the same situation is presented by rat and steer cells. The curves obtained with rat cells (FIGURE 8) shows that these cells, over a hemolyzing range of pH 5.5–6.7, are relatively stable compared to rabbit cells. Here, the total maximum thickness is 160 A.U., the leached thickness being about 90 A.U., thus, a volume ratio lipid to protein of about 0.8. Again, disruption of the envelope by the hemolyzing buffer indicates the envelope to be losing material of a fairly constant composition.

Steer cells (FIGURE 9) are considerably different from the others. In

these, the maximum thickness, at pH 6.0, is 180 A.U., the leached thickness being about 60.0 A.U., a volume ratio of lipid to protein of 2:1. In addition,

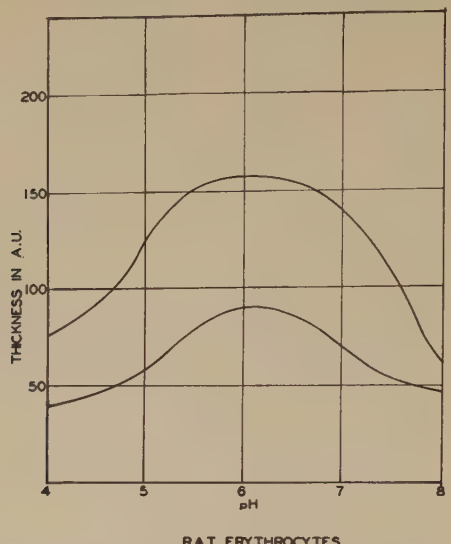


FIGURE 8. Thickness in Ångstrom units of erythrocyte membrane of rat cells versus pH of the hemolysing buffer.

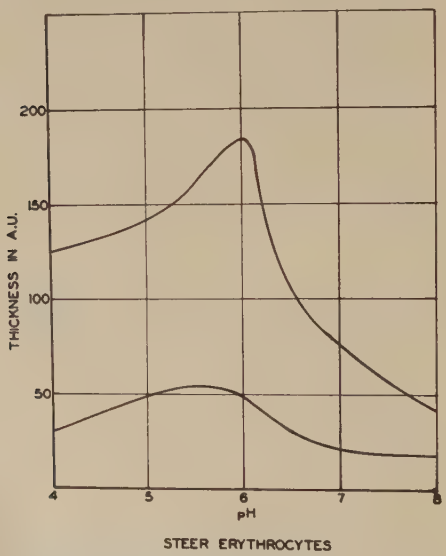


FIGURE 9. Thickness in Ångstrom units of erythrocyte membrane of steer cells versus pH of the hemolysing buffer.

steer cells are less resistant to alkaline buffers than to those on the acid side of the pH of maximum stability. This may be associated with the larger amount of lipid extractable material.

A summary of the data for rabbit, rat, and steer cells is shown in TABLE 1. It should be remembered that the recorded pH of maximal stability is that of the hemolyzing buffer. The pH in the neighborhood of the envelope may differ considerably from this value.

In the case of rabbit cells, on which more experimental work has been done than on the others, certain types of behavior are to be described.

As shown in the previous figures, the envelope is unstable in the absence of electrolyte. In the presence of isotonic saline, however, red cells may be buffered at pH of 5.0-8.0 without loss of material from the envelope. The

TABLE 1

THICKNESS AND COMPOSITION OF THE LIMITING ENVELOPE OF MAMMALIAN ERYTHROCYTES

Animal	*Maximal Thickness*						*Lipid: Protein Ratio*	
	pH	*Total Envelope*	*Lipid*		*Protein*		*By Volume*	*By Weight*
		AU	*AU*	*% of Total*	*AU*	*% of Total*		
Rat	5.7	160	80	50	80	50	1.0	0.60
Rabbit	6.1	215	135	63	80	37	1.7	1.01
Steer	6.1	180	125	69	55	31	2.3	1.36

TABLE 2

EFFECT OF ABSENCE OF ELECTROLYTES ON THE LIMITING ENVELOPES OF RABBIT ERYTHROCYTES

pH	*Time in hemolysing solution*	*Thickness*			*Refractive Index*	
		Total envelope	*Extracted envelope*	*Difference*	*Total envelope*	*Extracted envelope*
	min.	*A*	*A*	*A*		
6.1	1	232	131	101	1.504	1.525
6.1	2	196	113	83		
6.1	5	203	110	93		
6.1	10	146	86	60		
6.1	20	116	86	30	1.517	1.525
6.1	40	ca. 120	ca. 90	30		
			CO_2 Ghosts			
		130	100	30	1.520	1.525

addition of 10^{-5} M copper ion to the cells before and during hemolysis likewise reduces pH sensitivity. In addition, the ability to extract material with lipid solvents disappears. The copper evidently integrates the structure of the envelope by cross linking. Structural changes of this sort are probably the basis of the permeability changes found by Jacobs and Corson[17] and Davson and Danielli[18] for envelopes treated with heavy metal ions.

There is a general correlation between refractive index of envelopes, hemolysis time, and materials lost from the envelope at the hemolyzing pH of maximum thickness. TABLE 2 shows a summary of this information.

The first line refers to the standard technique. The envelopes have a thickness of 232 Å and a refractive index of 1.504. After leaching, the thickness decreases by 110 Å and the index of refraction rises to 1.525. Simple calculation indicates that the material leached out has a refractive index of 1.490, quite close to the refractive index for lipid materials. If cells are allowed to stand in the hemolyzing buffer for longer periods of time, the total thickness decreases and, at the same time, there is a preferential loss of low refractive index material from the envelope. The envelope is relatively stable with 30 Å of extractable material still present. During this time the extracted thicknesses decrease by 45 Å (Column 4), indicating the loss of protein. The envelope, air dried, is essentially a dehydrated structure, for, after several hours at 100°C, the thickness loss is less than 5 per cent. Thus, the thickness changes described are not due to changes in hydration.

The water content of the envelope *in vivo* is of more importance in determining permeability properties. The leptoscope adds information to this problem. Observation during drying and particularly just after recession of the water meniscus indicates that the envelope in the hemolyzing buffer, and probably therefore *in vivo*, is a structure containing *less* than 25 per cent water dispersed throughout its structure. This is in rough agreement with polarization optics. Thus, the *in vivo* envelope may be considered as a desolvated structure in which the component molecules are in close association.

In many instances, special structure may be seen in the envelope discs (see 12, Figure 2 B). Here, the central part of the discs reflects less light than its surroundings, indicating a thickening in this region. The difference in thickness is accentuated on leaching, showing that the thickening is due mainly to protein. One would expect this region to correspond to the regions of the biconcavities of the erythrocyte, for the cell adheres to the slide with a maximum surface in contact, thus with the concavities down. These protein thickenings in the regions of the biconcavities suggest that the shape of the cell is determined structurally in the envelope. The envelope, therefore, may have different molecular structures over its surface, not in the sense of a mosaic of submicroscopic regions but in the sense of regions, each of which constitutes a fair percentage of the entire cell surface.

Ponder in 1942[19] described the events leading to osmotic hemolysis and post-hemolysis phenomena as follows. The cells increase in volume, losing their biconcavities (stepwise?), and become spherical after an increase in volume of about 60 per cent. At this point, the hemoglobin escapes and the cells fade, but retain some hemoglobin. If the cells are now returned to isotonic saline, they shrink at first and then return to the biconcave disc structure. It seems improbable that internal strands of material, bridging the gap between the concave portions, could undergo such large changes in length in a reversible fashion.

Chemical Analysis. Chemical analyses of the lipid content of carefully washed hemolyzed cells, and in instances whole red cells, have been performed by a number of investigators (for a review see 20). With few exceptions, chemical analyses are in good agreement as to the total amount of

material present which may be extracted by organic solvents. The groups differ somewhat as to the distribution of this total lipid among the various lipid fractions, although the bulk of the material is accounted for by cephalin, cholesterol, and cholesterol esters. Dziemian[21] has made an extensive investigation of normal rabbit cells, Erickson *et al.*[3] have examined bovine and sheep cells, and Ponder[22] human erythrocytes. All agree that the content of lipid is sufficient to cover the surface of the cell with a layer about 40 Å thick. Erickson *et al.* have found only small amounts of lipid in the filtrate from hemolyzed cells, which is cited as evidence that no lipid is lost during hemolysis.

This value of 40 Å for rabbit cells (Dziemian) is to be compared with the 100 Å of lipid or extractable material found by the leptoscope. The difference is large enough to necessitate a careful re-examination of the materials which may be present in the envelope and of the various methods used to determine thickness. The leptoscope is currently being modified to give more accurate and completely objective analyses on single cells.

It is of consequence that the main phospholipid of the envelope is cephalin, a compound having a low iso-electric point and therefore capable of binding basic proteins with strong linkages, as Chargaff and co-workers[23] have shown.

The protein moiety has received attention. Jorpes[24] originally showed that the envelope protein from several sources contained 5.8 per cent arginine, 2.63 per cent histidine, 3.01 per cent tyrosine, and 1.46 per cent tryptophane on a dry weight basis. He found that the protein has a flocculation point at pH 5.5 and is therefore acidic. He concluded that the stroma-protein belonged to no well-defined group and therefore was a new type of protein. More recently Beach *et al.*[25] examined beef, sheep, hog, horse, and human erythrocytes. They found no significant differences between them, in agreement with Jorpes. Their analyses for beef cells gave: 1.9 per cent histidine, 5.1 per cent arginine, 3.5 per cent lysine, 2.9 per cent tyrosine, 1.2 per cent tryptophane, 0.78 per cent cystine, 1.5 per cent methionine, and 0.708 per cent total sulfur. To these values we may add those of Ballentine[26] for beef as follows: 3.66 per cent glycine, 10.7 per cent leucine, 3.42 per cent tyrosine, 1.45 per cent tryptophane. Ballentine also examined sheep and human cells and found no appreciable differences. From the content of leucine and glycine, he agrees with Jorpes that stromatin represents a new type of protein at present peculiar to the erythrocyte envelope. The possibility that more soluble proteins are being washed out during preparation is recognized.

Chemical analyses agree, therefore, that the stromatin recovered from a number of mammalian species after prolonged washing has a relatively constant composition and probably represents a characteristic envelope protein. A correlation between permeability properties and the lipid or protein content of the envelope has not been made.

Rough calculations, based on Jorpes'[24] calculations that about 4 per cent of the total erythrocyte protein is stroma protein and Ballentine's[26] recovery of 14–16 gms. stromatin from 2 liters of packed washed cells, indicate that

the thickness of the protein component of the washed envelope is approximately 70 Å.

Status of Molecules within Membranes. We have seen that the results of polarized light and electron microscope investigations suggest the protein component to be in the form of platelets, each platelet being composed, in turn, of a feltwork of filaments or fibrils. The average thickness and lateral domain of these platelets remains to be determined. The lipid molecules also remain undetermined in the sense that we know only that they must be radially oriented and that they probably exist in groups to account for the intrinsic double refraction. The unique properties of the envelope may be encompassed in a structure having a maximum dehydrated thickness of 215 Å and a hydrated thickness only about 25 per cent more than this value. Further insight into the problem may be gained, however, if we admit evidence from other sources as bearing on the problem. A careful investigation of the structure of the nerve myelin sheath, a lipid protein complex, has been made by F. O. Schmitt, W. J. Schmidt, R. S. Bear, K. Palmer, and others (see Reference 1), using polarized light and X-ray diffraction techniques. The situation may be summarized as follows: Polarization optics show the sheath to be constructed of concentric layers of protein with layers of lipid in between. The lipid molecules have their long axes radially oriented. X-ray diffraction evidence shows, in the radial direction, a fundamental repeat period of 170–180 A.U., with other spacings of 4.7 and 9.4 A.U. considered as due to the lipid side chains and whole molecules. These latter spacings suggest that the lipid molecules are closely packed in planes and exist as a mixed phase. The long spacing of 170–180 A.U. is sufficient to accommodate approximately two double layers of lipid, leaving 25 Å for protein. The position of the protein in nerve has not been determined. It may exist in between each double layer of lipid or, more probably, in between each second double layer of lipid.

The normal association of polar groups with polar groups and hydrophobic groups with hydrophobic groups causes lipid to associate, typically, in double layers. This tendency is evidenced strikingly in built-up films, as shown previously, and is evidenced also in emulsions of several varieties of lipid molecules. Hess, Philippoff and Kiessig, Stauff, and Kiessig and Philippoff,[27] working with sodium oleate solutions, and Schmitt and Palmer,[28] with nerve and other lipid, found this to be a general rule. Indeed, the double layer structure is maintained even though the planes of polar groups are expanded by intercalation of water[27, 28] or the planes of the hydrophobic residues are moved apart by the intercalation of organic solvents.[27c] In the former case a condensed structure with polar groups in close apposition may be restored by the addition of 0.6 M KCl or as little as 0.04 M $CaCl_2$.[28] The striking effect of calcium is probably due to its ability to bind polar groups of of two contiguous layers. As Schmitt and Palmer point out, the presence of such salts in biologic systems would cause the lipid layers to be held in close association with a minimal amount of water between them.

On the basis of such evidence, it seems reasonable to suppose that the lipid of the erythrocyte envelope is present, at least in local regions, in condensed layers, *possibly* double layers. The structure of the envelope may

not, however, conform to that found for the myelin sheath of nerve. The questions associated with mosaic structure, uniform complexes, *etc.*, are as yet unanswered.

Monomolecular Films. Evidence drawn from monomolecular film work indicates certain molecular mechanisms which may play an important part in determining permeability properties of cells and foreshadows the possible degree of complexity with which ultrastructure determination may have to deal. For some time, it has been known that a compressed film of stearic acid on a water surface at low pH produced little measurable retardation of evaporation of water, while a film of cetyl alcohol would reduce evaporation by one half. Langmuir and Schaefer[29] have recently analyzed the process in considerable detail. They suspend a drying agent over the film and measure the increase in weight of the drier with time, thus enabling a quantitative calculation of the resistance offered by the film. Langmuir and Schaefer show that the presence of the film probably introduces an energy barrier to evaporation. The extent of this energy barrier is largely determined by the internal stresses which occur within the monolayer. As they point out, the water surface holds the polar groups rigidly within a plane, the hydrocarbon chains lying all on one side of the plane. The hydrocarbon chains have minimal energy values for specific angles of tilt with respect to the basal plane. From the known properties of hydrocarbons these angles are 90°, 63° 10′, and 44° 40′. At the same time, the areas occupied at the chain ends are correspondingly 18.5, 20.51, and 26.0 Å^2. Between the minima in potential energy corresponding to these areas must be maxima.

The polar groups tend to spread out over the surface, while a contracting force is represented by the attractive forces between hydrocarbon tails. The measured surface pressure is the resultant of these forces.

The area per molecule must be the same for head and tail. If the heads are forced apart and must occupy areas somewhat larger than those corresponding to minima, *e.g.*, 20.5 Å^2, the tails must do likewise. This increase in area must give rise to large contracting forces which oppose the increase in area. Langmuir and Schaefer show, however, that this effect alone is not sufficient to account for the energy barrier. The remainder is to be accounted for by the crystal-like properties of condensed monolayers. The presence of a water molecule between the heads causes a local breakdown in crystal structure which affects a relatively large number of neighboring molecules. Many molecules co-operate in producing the barrier and in this way the tremendous forces opposing passage may be accounted for.

The layers of lipid molecules in cellular elements have a structure corresponding closely to that of condensed films on water. One would expect the same general principles, modified as to detail, to apply in both cases. Thus one might conclude that the energy barrier associated with permeability[30] is due to the specific packing of the component molecules (protein as well as lipid). It is likely that this packing is determined with great precision to account for the specificities involved.

It is interesting to note that the great resistance to evaporation offered by cetyl alcohol monolayers is profoundly altered by the presence of minute

amounts of materials having a much lower resistance. The obvious inference is that small amounts of surface active materials change cellular permeability by a similar mechanism.

The greater part of the discussion presented has been drawn from material now several years old. The author is aware that no mention has been made of the dynamic aspect of the cell surface, the changes in structure which may occur momentarily and which are undoubtedly integrated with the simultaneous activity of the cell. We can hope that new instruments and techniques will be developed in the near future for handling the next phase of ultrastructure determination: the many problems associated with the interaction of molecules, particularly those interactions which depend upon specific intermolecular forces. This end may best be accomplished by integrating information coming from biochemistry and the application of physical tools to biological problems.

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Discussion of the Paper

Dr. Maurice M. Black (*Brooklyn, New York*): Polarization optics, leptoscopic examination, and electron microscopy constitute valuable tools in the elucidation of the structural organization of biological material. However, it should be kept in mind that manipulative procedures incident to these modalities may often introduce extensive artifacts. Thus, the discrepancies in the determination of the structure of the red-cell membrane by different investigators using somewhat different methods may be explained on alterations incident to examination. The lability of lipo-protein systems is well demonstrated in the myelin sheath of nerve fibers. I have done some work on isolated peripheral nerves[1, 2] and find that immersion of freshly prepared nerve segments in water or non-electrolytes results in a swelling of the myelin and movement of the latter out of the cut ends of the fiber. This is associated with a loss of the birefringence of the myelin sheath. A definite relation exists between the pH of the solution and the amount of the reaction observed, no visible reaction occurring at pH 4.8 but increasing to maxima at the extremes of the pH scale. Instability of the lipo-protein complex is further demonstrated by the fact that undue handling causes myelin-forms to be produced in the sheath. Moreover, the lipo-protein orientation is upset as evidenced by the fact that the handled parts do not possess the double refraction found in the rest of the sheath. Thus, it is clear that lipo-protein orientation must be in an extremely delicate balance. Similar alteration in structural orientation was found in the axis cylinder, where even such slight injury as produced by immersion for several hours in isosmotic saline solution will result in loss of previously demonstrable neurofibril structure. Every one agrees that such lability must be considered a limiting factor in measurements designed to evaluate structural relations in biological material.

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Dr. Rudolf Höber (*Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pa.*): It is suggestive to test with erythrocytes the pore theory and the solvent theory of permeability by measuring the membrane potentials. The erythrocytes of some Amphibia (*e.g.* Proteus) have a diameter up to 50 to 70 μ. This is comparable to the thickness of single muscle fibers of frog (70 μ) which have been shown by Graham and Gerard to allow puncturing of the plasma membrane with capillary microelectrodes (5 μ). Possibly it will be very useful to study in this manner the effect of neutral non-toxic, non-polar-polar organic sodium salts having anions of various sizes.

Dr. Eric Ponder (*Nassau Hospital, Mineola, New York*): It is very satisfactory to workers in the field to see, from the contents of Dr. Jacobs' and of Dr. Waugh's papers, that there is so much agreement regarding the essential architecture and properties of the red-cell surface ultrastructure. It seem to me, however, that the old and still not wholly settled controversy, as to whether the mammalian red cell is a balloon-like body with a fluid interior or a gelatin-lozenge-like body with an internal structure, is reappearing in a new form. For we now have to ask ourselves: How do we know that some of the properties which we ascribe to the surface, such as some of the permeability properties, are not really surface properties as modified by the properties of the cell interior, or even properties largely dependent on what is on the inside of the surface ultrastructure?

In the case of many changes which happen rapidly, such as the transfer of water, of anions, and of many organic substances, and also in the case of the volume changes which these rapid transfers produce, the effects of the interior can probably be ignored, because it is likely that they enter into the equations which describe these rapid happenings only in such a way as to change the values of constants. There are situations, however, in which there is evidence that the interior of the cell, with its closely packed and probably oriented Hb molecules, plays an important part in the phenomena observed. The departure from ideal osmotic behavior shown by crenated red cells seems to be associated with the paracrystalline state of their Hb, and this may be so extreme, as in the rat red cell which has been kept in isotonic citrate in the cold, that the cells do not hemolyze or even swell when placed in distilled water. Another phenomenon, which is probably dependent on forces associated with the cell interior, is the slow escape of cations from red cells which have been exposed to hypolytic concentrations of lysins, and particularly to hypolytic concentrations of alcohols such as resorcinol, in which human red cells may exchange more than 50 p.c. of their K for Na and yet undergo less than a 10 p.c. increase in volume. It is true that these phenomena are observed only under special conditions, but they, as well as the properties of normal red cells, have to be accounted for in any complete description of red-cell structure. Otherwise, the description would be like a description of the structure of muscle which could account for the resting state and for contraction, but not for a phenomenon such as contracture.

It seems to me that it is possible to accept most of the information regarding the properties of the red-cell surface which has been accumulated, but not to be content with the description until a fuller account has been taken of the group of phenomena which seem to depend on the properties of the material which the surface ultrastructure encloses. Until matters are clarified, one should not allow one's self to think that when one measures dQ/dt, the rate of passage of a substance across the surface of the cell, one is necessarily measuring permeability properties confined to a membrane or even to a surface ultrastructure. It is possible that in some cases one is measuring the rate at which a material distributes itself between the suspension medium as one phase and the material of the cell surface plus the material of the cell interior as another phase, without the measurement's having any simple relation to membrane permeability as ordinarily understood.

Dr. Chambers' reference to a surface structure which may have no inner boundary, but which passes into the structure of a cytoplasm within it, expresses an idea which appeals to me strongly, as also does his description of the general nature of the cytolytic process. A process of the same kind may be involved in lysis of the red cell. Wilbrandt's colloid-osmotic hemolysis, which is similar to the "dual mechanism" hypothesis which Davson and I advanced in 1938, is certainly not satisfactory as a complete account of what happens. Both the colloid-osmotic hypothesis and the dual mechanism suppose that lysins render the red cell permeable to cations, and that this is necessarily followed by a secondary Donnan swelling of the cell. There are many instances, however, in which mammalian red cells slowly become permeable to both K and Na, but in which large ionic exchanges occur without more than a small volume increase and without loss of stability, *i.e.*, without the secondary Donnan swelling. One gets the impression that the full effects of the osmotic pressure of the intracellular Hb are not observed until some new event takes place. This event probably follows the K-Na exchange and is an immediate antecedent of lysis. It may be a cytolytic process, initiated by the collapse of the surface ultrastructure, and spreading through an interior composed of oriented Hb and stromatin molecules.

STUDIES ON CELL MORPHOLOGY AND FUNCTIONS: METHODS AND RESULTS

By Albert Claude*

The Laboratories of the Rockefeller Institute for Medical Research, New York, N. Y.

To a large extent, our present knowledge of cell morphology has derived from observations of cells and tissues under the microscope, but inquiries by the microscopical method alone have repeatedly failed to give conclusive information on the chemical constitution of the various cell structures and the topography of biochemical functions.

The chief difficulties inherent in histochemical methods are: (1) the chemical tests which otherwise may be specific must be carried out on cell structures altered by fixation and dehydration to an extent which cannot be ascertained; (2) the chemical reactions studied must take place on structures, and in surroundings, of unknown and uncontrollable complexity; and (3) at the microscopical level, one must deal with exceedingly small amounts of substances, which often places the tests beyond the sensitivity of the reaction to be employed.

Since Miescher, attempts have been made to apply analytical methods to the study of cell structures and to isolate known cell components mechanically or through mild chemical manipulations. Separation of nuclei led to the discovery of nucleic acids and the recognition of certain basic proteins.[1] In 1913, Warburg segregated cytoplasmic granules by centrifugation and was able to show that these were responsible for most of the oxygen uptake of cell-free extracts of guinea-pig liver.[2] In 1934, Bensley and Hoerr succeeded in separating, also by centrifugation, large cellular elements which they identified as mitochondria.[3] In 1938, submicroscopic components of the ground substance were isolated by means of centrifugation at high speed.[4] These results indicated that, given the proper equipment and a systematic application of the method of differential centrifugation, it would be possible to separate the various cell components by mechanical means.[5–8]

In this paper, I shall attempt to review and discuss briefly the usefulness and the difficulties of the method.

The Method of Differential Centrifugation. The method of fractionation by differential centrifugation, as applied to cell studies, precisely overcomes the most serious limitations of the microscopical technique. By means of centrifugation, the whole range of particle and molecular sizes can be investigated, since centrifuges can be made to provide centrifugal fields of practically any given strength. In practice, however, the use of very high centrifugal force is not necessary. Under ordinary conditions, *i.e.*, when the viscosity and density of the medium is approximately that of water or saline, the cellular components with a diameter equal to, or greater than, 50 μ can be separated completely by centrifugation of one hour at 20,000 xg. In this manner, particulate components of cytoplasm (microsomes) too small to be detected by light microscopy could be demonstrated and isolated in

*Present address: Institut Jules Bordet, 1 Rue Heger Bordet, Brussels, Belgium.

bulk.[4, 7] The second outstanding advantage of the method of differential centrifugation is that cell components can be obtained in practically unlimited amounts, thus making it possible to investigate their constitution and activities by the use of current chemical and biochemical methods.

Providing that the separation of the cell elements has been effective, a fact to be ascertained by suitable microscopical methods, the observations cannot take their full significance, and indeed can be misleading, unless the chemical and biochemical tests are carried out on a strict quantitative basis and the values pertaining to each individual fraction estimated in terms of the total capacity of the unfractionated cell and that of the other cell portions. For example, the microsome fraction was found to exhibit certain enzymatic activities (catalase; succinoxidase), but these had to be considered negligible in view of the considerably greater activity possessed by the whole suspension and by the large granule fraction.[9] Likewise, the distribution of ribose nucleic acid should be re-examined on a more quantitative basis, since ribose nucleotides are among the most common constituents of cells and, without special care, may be found in every fraction isolated.

Three Main Cytoplasmic Fractions. Under given conditions, it is possible to break a certain proportion of tissue cells while the free nuclei remain intact.[8] The debris, unbroken cells, and free nuclei can then be removed by low-speed centrifugation, leaving a suspension which contains essentially the components of the cytoplasm. By centrifugation at appropriate speeds, this "cytoplasmic extract" can be fractionated, stepwise, into three main portions: (a) a *large granule* fraction composed of elements of relatively large size, *i.e.*, ranging roughly from 0.5 to 2.0 μ in diameter; (b) a *microsome* fraction consisting in "sub-microscopic" elements approximately 50 to 200 mμ in diameter; and (c) the *supernate*, left after the two preceding fractions have been removed. This fraction contains the constituents of the cytoplasmic extract not sedimentable at the speed employed and, therefore, inferior in diameter to 50 mμ.[8]

It must be pointed out that the division of the cytoplasmic extract in this manner is not arbitrary and is not based on size differences alone but is determined by the fact that each of the three fractions isolated possesses physical properties, a chemical constitution, and biochemical activities of its own.

The so-called large granule fraction consists of small elements limited by what appears to be a semi-permeable membrane, which is found to respond osmotically to variations in the salt concentration of the medium. Thus, the large granules can be observed to swell or shrink when the salt concentration is lowered or increased.[7] The existence of a membrane has been demonstrated by electron microscopy.[10, 11] When concentrated in the centrifuge, the large granules give a mass which is opaque, brown, or distinctly yellow.[3, 12] Chemically, the major constituents of the large granules are proteins, phospholipids, ribose nucleic acid, ribose nucleotides, and flavins. The most distinctive feature, however, is that the large granules contain a number of enzymes and enzyme systems which are not found in the other cytoplasmic fractions. For example, the enzyme d-amino acid oxidase and the corresponding co-enzyme were found to be associated entirely with the

large granules, the other two fractions exhibiting no activity. Likewise, conclusive evidence has been obtained which indicates that important parts of the respiratory system, namely, cytochrome oxidase, succinoxidase, and cytochrome c, also reside in the large granule fraction.[9, 13, 14, 15]

The microsomes, on the other hand, are submicroscopic elements which have been shown to constitute the chromophilic component of the cytoplasmic ground substance.[7] Whether these elements possess a limiting membrane, like mitochondria, has not been demonstrated, but they become more hydrated and more finely dispersed when placed in hypotonic salt solutions or in distilled water.[8] In the pellet of centrifugation, the microsomes form a perfectly transparent mass, which may be brown or red in color when obtained from liver and colorless when derived from lymphoid tissues or pancreas.[8] The microsomes are complex structures, the major constituents detected, so far, being phospholipids, proteins, and ribose nucleic acid. In contrast with the large granules, no enzymatic functions have been found associated exclusively with the microsomes, although these cell constituents have been shown to possess high thromboplastic activity.[16]

The supernate is perhaps less characteristic than the two other cytoplasmic fractions. It contains proteins, "soluble" enzymes, some ribose nucleic acid, nucleotides, and organic and inorganic compounds of small molecular weight. It is not certain to what extent some of these constituents may have derived from mechanical or enzymatic breakdown of other cell elements during preparation of the extract.

As already pointed out, a separation of the major morphological constituents of the cell can be accomplished by means of centrifugation because of the pronounced differences in sizes which exist between the various groups or families of cellular elements. Whole cells in suspensions can be separated readily from the free cell components, since the next in size, the nucleus, represents no more than 6 to 10 per cent of the volume of the cell. Chromosomes are significantly smaller than nuclei and, in turn, are considerably larger than mitochondria or secretory granules. Similarly, a large gap exists between mitochondria and microsomes and, again, between microsomes and the elements of the supernate.

Similar segregation of the cytoplasmic components into three fractions resembling those just described has been obtained by submitting various types of tissues, namely, liver, pancreas, spleen, lung, mammary gland, embryos, and a number of tumors, to differential centrifugation. It should not be inferred, however, that each of these fractions, especially the large granules and the microsomes, are represented by a single kind of element.

Complexity of the Main Cytoplasmic Fractions. Evidence exists which indicates that the large granule fraction, at least, is composed of a variety of elements which are different in constitution and, especially, in functions.

Prominent components of the large granule fraction are the mitochondria. These elements are generally recognized because of their shape, often filamentous or rod-like, and their location in the cell, and for certain staining reactions which, however, are not entirely specific. In embryo, and in undifferentiated tumor cells, mitochondria appear to represent the major part of the large granules which are detectable in the cytoplasm by ordinary

microscopical methods. The large granule fraction derived from these cells, therefore, can be considered to be composed essentially of mitochondria.[20]

The situation is obviously different in cells and organs where several types of large granules are detected microscopically in the cytoplasm and where the cells are known to be engaged in a variety of highly specialized functions. This is the case for the pancreas, where zymogen granules and mitochondria are found in about equal proportions. The case of the liver is somewhat more complex. It has been shown that liver cells contain at least two types of granules, *i.e.*, mitochondria together with secretory granules which are expelled at the time of feeding, a situation very similar to that found in the pancreas.[8] It may be assumed, however, that the liver granules are even more diverse, and it is possible that each of the various liver functions is segregated in different, specialized elements. The problem would be further complicated if, as it has been suggested, secretory granules derive from the progressive transformation of mitochondria or are produced by them.

A separation of granules of nearly the same size is possible when these happen to differ markedly in specific gravity. Thus, the dense melanin granules found in amphibian liver could be separated readily from mitochondria although they are approximately of the same size and shape.[5] Recently, it has been possible to fractionate by centrifugation the large granules of rat liver into "heavy" and "light" portions, the former exhibiting about twice the succinoxidase activity of the latter.[17]

Fractionation of Large Granules. The method of differential centrifugation can be applied not only to the separation of various cell components but also to the fractionation of some of the cell components so isolated.

As already noted, the large granules can be separated and washed repeatedly in saline. When placed in distilled water, these elements swell, in the manner of red cells under similar conditions, and finally disintegrate. By means of high-speed centrifugation, it is possible to separate from the suspension a particulate component of submicroscopic size. This small component of large granules has been found to have associated with it most of the ribose nucleic acid originally present in the large granules.[8] The supernate from this high-speed centrifugation was found to contain proteins (among them, undoubtedly, enzymes), ribose nucleotides, and flavins. These two groups of substances of widely different molecular weights can be separated by dialysis.[8] In this manner, some of the morphological features of the large granules were revealed. The presence of small elements, approximately 0.1 μ in diameter, detected in mitochondria by electron microscopy,[10] seems to confirm the observations just mentioned.

Isolation of Chromatin Threads. The isolation of chromatin threads from the resting nucleus was first reported in December, 1941.[5] The elements which had been separated were stained specifically with the Feulgen technique and, on analysis, were found to contain as much as 40 per cent of desoxyribose nucleic acid. These findings, together with the frequent occurrence of double strands and beaded structures, suggested that the elements isolated represented chromosomes or were derived from them.[6] This method for the mechanical isolation of chromosomal material has been

adopted by Mirsky and his associates and used extensively since then to extract nucleoproteins and study the chemical composition of chromosomes.[18]

Distribution of Cell Functions. Microscopical studies, and especially the isolation and *in vitro* analysis of various cell components, have provided conclusive evidence that a cell is not a continuum but is composed of a number of units which are distinct in sizes, chemical composition, and functions. It is the sum of the activities and interactions of these various entities which accounts for the life of the cell. The concept that certain morphological constituents of the cell, such as mitochondria, are reversible systems which could disappear or be created *de novo* in the cytoplasm (coacervates) does not seem to be supported by the recent findings. For example, desoxyribose nucleic acid is found in abundance in chromosomes[6] but, so far, has not been detected anywhere else in the cell. Extensive studies have shown that these bodies of unique chemical composition are the site of segregation of genetic characters. The mechanism by which chromosomes, through individual genes, act to influence the constitution and activities of the rest of the cell is not yet understood. It can be hoped, however, that the possibility of isolating chromosomes, and their analysis *in vitro*, will aid effectively in solving this basic problem of cytology.

Mitochondria appear as the most conspicuous constituents of the cell, next to the nucleus and chromosomes, because of their abundance and typical morphology and behavior. Mitochondria are universally found in active cells, and there is no doubt that they constitute one of the basic building stones of protoplasm. Filamentous or rod-shaped, they are, as a rule, of remarkably constant and uniform width. They are probably self-duplication elements. Unlike chromosomes, however, they do not divide lengthwise but appear to grow by elongation, dividing across their length. Passive division of mitocondria can be clearly observed during mitosis of certain cells, such as the germ cells of the grasshopper and the Arizona *Triton*.[19] Mitochondria have been found to represent an important portion, in dry weight as much as 15 to 20 per cent, of the cell mass.[20, 8] Chemically, mitochondria are characterized by the presence of phospholipids, which probably enter in the constitution of the limiting membrane, and they are the site of important enzymatic activities.[9] As already stated, d-amino acid oxidase activity has been found to be associated with the so-called large granules but not with the other cytoplasmic fractions. It has been found that d-amino acid oxidase activity could be progressively reduced by repeated washing of the granules and restored by the addition of co-factors in the form of heated yeast extract. These results indicate that the two components of the enzyme are both present in the large granules, although one of them is slowly diffusible.[21] Similarly, investigations during the past few years in different laboratories[13, 14] have provided conclusive evidence demonstrating that important members of the respiratory system, namely, cytochrome oxidase and succinoxidase, are located entirely in the large granules. Recent experiments have shown, further, that cytochrome c, a readily soluble component of the system, is also found in the large granules and can be released from them under certain conditions.[15] These observations indicate that the power of respiration is not diffusely distributed throughout the

cell but is probably segregated, together with other enzymatic function, in the formed elements referred to under the general term, large granules. Mitochondria, and the large granules generally, can then be visualized as representing the actual power plants of the cell, where the energy of molecular oxygen is ultimately transferred and utilized. Since it is probable that some of the synthetic activities of the cell take place near the source of energy, it may be assumed that the large granules also represent important manufacturing centers. The findings would support the view, frequently held, that secretory granules are but transformed mitochondria, or are parts of mitochondria, loaded with the accumulated products of their metabolic activity.

In a series of papers, Caspersson and Brachet have developed the view that ribose nucleic acid is related in some way to protein synthesis.[22] This opinion was based, first, on the observation that ribose nucleic acid is always present in the cytoplasm of metabolically active cells and, second, on the fact that ribose nucleic acid is found in greatest abundance in actively growing cells such as embryonic and tumor cells, where synthesis of proteins is presumably needed, and in cells primarily engaged in the production and excretion of proteins, as in the pancreas. The ribose nucleic acid detected by the ultraviolet method or by staining appears to occur in a diffuse form in the cytoplasm (ergastoplasm). On the other hand, observations from this laboratory have shown that most of the ribose nucleic acid of the cytoplasmic ground substance occurs in association with particulate elements of submicroscopic size, the microsomes.[7] If the ribose nucleic acid of the microsomes is to play a role in protein synthesis, it must be without the immediate participation of the cytochrome-linked respiratory system, since cytochrome oxidase, succinoxidase, and probably cytochrome c are absent. On the other hand, the role attributed to ribose nucleic acid as a factor in protein synthesis is not the only hypothesis that could be held to account for the abundance of this substance in embryonic and tumor cells, since its presence coincides with other outstanding functions of these cells. It may be noted that, in general, the cells which have been found to have a high ribose nucleic acid content, such as embryonic and tumor cells, have been shown by Warburg and his followers to possess to a high degree the power of anaerobic glycolysis. It might be suggested, therefore, that the presence of ribose nucleic acid in cells is related not directly to protein synthesis, but to their capacity for anaerobic respiration. This view would seem to be supported by the concurrence of large amounts of ribose nucleic acid, and of active fermentative processes, in yeasts and in certain bacteria.

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THE LOCALIZATION AND THE ROLE OF RIBONUCLEIC ACID IN THE CELL

By Jean Brachet
University of Brussels, Belgium, and University of Pennsylvania, Philadelphia

Methods for the Cytochemical Detection of Ribonucleic Acid. The chemical properties of ribonucleic acid, so far as cytochemical detection and microestimation are concerned, depend on: (a) orthophosphoric acid, (b) *d*-ribose, an ordinary pentose, and (c) purine and pyrimidine bases.

The presence of phosphoric acid in nucleic acid molecules confers on them a strong affinity for basic dyes. As a rule, basophilic structures in the cell contain either ribo- or desoxyribonucleic acid. Glycoproteins, also strongly acidic, are usually easy to recognize because of their metachromatic properties.

Ribo- and desoxyribonucleic acids can be distinguished by the fact that only the latter gives a positive Feulgen test, a reaction due to the presence of *d*-2-desoxyribose. Furthermore, nucleases which attack either ribo- or desoxyribonucleic acids are easy to prepare. A useful method for the detection of ribonucleic acid is to treat microscopic sections with ribonuclease. Structures which contained ribonucleic acid before the enzymatic digestion fail to stain with a basic dye, such as toluidine blue, after this treatment. Similar digestion with desoxyribonuclease (thymonucleodepolymerase) results in a negative Feulgen test, as well as the disappearance of basophilia in the chromatin. An interesting combination of basic dyes is Unna's methyl green-pyronine mixture which, as a rule, stains ribonucleic acid-containing elements red and desoxyribonucleic acid-containing elements green. After digestion with ribonuclease, the sections stain only green.

The value of the action of nucleases as a test is, of course, limited by the specificity of the enzymes used. There have been various reports (Schultz,[36] Mazia,[31] Cohen,[21] and Gersh and Bodian[26]) that crystalline ribonuclease has a definite proteolytic activity, which, however, varies strongly from one sample to another (Schneider[35]). According to Baker and Sanders,[1] heating the ribonuclease in a slightly alkaline medium entirely destroys the proteolytic activity without harming the ribonuclease activity. Furthermore, we recently found (Brachet and Shaver[34]) that a crystalline ribonuclease preparation, kindly supplied by Dr. M. Kunitz, had no observable proteolytic activity when sections were treated with the enzyme. After treatment with the ribonuclease, the sections showed no appreciable decrease in the intensity of arginine and tyrosine when subjected to the tests recently described by Serra.[37] These cytochemical tests for amino acids, as used in these experiments, provide a simple method to check the actual proteolytic activity of a given preparation of ribonuclease, in conditions identical to those prevailing for the ribonuclease test itself.

The presence of ribose in the ribonucleic acid molecule cannot, so far, be used for the cytochemical detection of this acid, with the possible exception of Turchini's[41] method (staining of the hydrolyzed sections with a fluorine

derivative), which was reported to give a violet color with desoxyribonucleic acid and an orange color with ribonucleic acid. The transformation of ribose into furfural by acid hydrolysis affords convenient and sensitive methods for the micro-estimation of ribonucleic acid. These methods are valuable, since they often give an opportunity to check the cytochemical observations.

Finally, the strong selective absorption in the ultra-violet band around 2600 Å is the basis of the very fine method of Caspersson[11, 12] for the detection of nucleic acids. Ribose and desoxyribose nucleic acids have nearly identical absorption curves. If both have the same localization in the cell, Caspersson's methods cannot be used to differentiate between them. The combined use of basic dyes, ultra-violet absorption, and the nuclease tests constitutes the best cytochemical method for the detection of nucleic acid now available (Gersh and Bodian[26] and Davidson and Waymouth[23]).

The Localization of Ribonucleic Acid in the Cell. Results obtained independently by the ultra-violet absorption method (Caspersson[11, 12]) and by the ribonuclease technique (Brachet[7, 8]) agree perfectly in showing that ribonucleic acid is present in the nucleolus and in the basophilic parts of the cytoplasm, the so-called ergastoplasm. Staining with Unna's mixture combined with ribonuclease digestion indicates, furthermore, the presence of small amounts of ribonucleic acid in chromatin, especially the heterochromatin. This result was corroborated by analysis of isolated nucleic, where 10 per cent of the total nucleic acid was in the form of ribonucleic acid (Brachet[7, 8]).

The amount of basophilic, ultra-violet absorbing material varies considerably from one type of cell to another. Little of it is present in physiologically active organs such as heart, kidney, and striated or smooth muscles. On the other hand, large amounts of ribonucleic acid are present in the exocrine pancreas, the pepsin-secreting cells of the gastric mucosa (excluding the chloride-secreting cells), actively dividing cells from the adult or from the embryo, growing oöcytes, and neurones.

The distribution of ribonucleic acid in various cells and tissues has been further confirmed by micro-chemical quantitative estimation of the ribonucleic acid content of various organs (Brachet[8] and Davidson and Waymouth[23]), which can be arranged in a series, parallel to the basophilia, as follows: pancreas > intestinal and gastric mucosa > liver > spleen > lymph nodes, testis > kidney, muscle, heart, lung. The strongly basophilic young oöcytes of the frog contained about three times more ribonucleic acid per mg. than the large weakly-staining ones. The same relationship was seen on comparing the gastric mucosa with the non-basophilic muscularis.

The peculiar distribution of ribonucleic acid suggests strongly, as pointed out by Caspersson[11, 12] and by myself,[6, 7] that some link must exist between this acid and protein synthesis. The pancreas and the gastric and intestinal mucosa secrete large amounts of enzymatic proteins, while the growing oöcyte is, of course, the site of a tremendous elaboration of proteic yolk. Dividing cells must synthesize their own substances. As for the neurones, it has been shown recently by Weiss[42] that the nerve fibers are the site of a continuous proximo-distal growth and that the protoplasm surrounding the

nucleus of the neurones is continually synthesizing itself. Further evidence for the view that ribonucleic acid is concerned with protein synthesis lies in the fact that the cells in the silk gland of the silkworm caterpillar are, to the best of my knowledge, the richest in ribonucleic acid. The only known function of these cells is the secretion of the protein, silk.

Furthermore, it is interesting to note that changes in the ribonucleic acid content of certain cells have been observed in experimental circumstances which are known to affect protein synthesis. For instance, stimulation of pituitary activity by the injection of estrone (Desclin[24]) or pregnancy hormones (Herlant[28]) led to an increase in the ribonucleic acid content of the gland. By contrast, the prolonged starvation in rats produces a decrease in the ribonucleic acid content of the liver just at the time when it is known that protein synthesis by the liver is no longer possible (Brachet, Jeener, Rosseel, and Thonet[10]). Comparable observations have been reported by Caspersson.[11, 12]

When silkworms contract a virus infection, the so-called jaundice disease, the virus accumulates in the nuclei of the fat body cells in the form of polyhedrons. The first reaction to the infection is an increase in the perinuclear basophilia, together with a striking increase in cell size. The virus thus induces a ribonucleic acid synthesis in the infected cells, followed shortly by growth and protein synthesis (Gratia, Brachet, and Jeener[27]).

It is worth adding that the cytoplasm of cells in the actual process of mitosis usually is much less basophilic than that of adjoining, non-dividing cells. Such observations agree with our previous observation[6] on nucleic acid synthesis in sea-urchin eggs, where quantitative estimation of both types of nucleic acids strongly supports the view that ribonucleic acid can be transformed into desoxyribonucleic acid. Such a transformation is, according to Mitchell,[32] prevented by X radiation of dividing cells, which leads to an accumulation of ribonucleic acid in the cytoplasm. It should, however, be pointed out that, during embryonic development, two antagonistic processes are going on almost simultaneously. One of them is a decrease of the ribonucleic acid content of individual cells at the time of division. The other is the production of the same acid when new proteins are synthesized as a result of organogenesis, differentiation, and growth. Close links between the synthesis of ribonucleic acid and protein during development have been observed in the embryos both of Amphibia (Brachet[7, 8]) and of the chick (Caspersson and Thorell[14]).

The Constitution of the Cytoplasmic Ribonucleoprotein Granules. While cytochemical observations strongly suggest a link between ribonucleic acid and protein synthesis, there is no available biochemical evidence in favor of this view. We have, as yet, no indication of the mechanisms by which ribonucleic acid might be an agent in the synthesis of proteins.

Since Claude's[17] work indicated, as early as 1939, that ribonucleic acid is a constituent of cytoplasmic granules which can be isolated by high speed centrifugation, it became important to know more about the chemical composition and the role of these particles. In most of the work which was carried on at the University of Brussels by Jeener, Chantrenne, and myself, no attempt was made, owing to war difficulties and lack of equipment, to

separate the granules into various fractions. The tissues were crushed with sand and extracted with 10 volumes of M/200 phosphate buffer at pH 7.3, and centrifuged at low speed (1500 G.) for 10 minutes. The supernatant fluid was ultracentrifuged for 10 to 20 minutes at 100,000 G. in an air-driven ultracentrifuge. The pellet, which contained both *large granule* and *microsome* fractions (Claude[18, 19, 20]), was used for comparison with the supernatant fluid from the ultracentrifugation. In more recent work by Chantrenne,[16] which will be described later, several fractions, instead of two, have been isolated by differential centrifugation at various speeds.

The granules, a mixture of Claude's[20] mitochondrial and microsomal fractions, could be isolated without difficulty from all vertebrate or invertebrate tissues studied. In all instances, they showed similarities in their chemical constitution, since they always gave positive tests for the following substances: indophenol oxidase, peroxidase, -SH groups, plasmalogen, and ribonucleic acid. The relative intensity of these tests varied from one organ to another, however, and quantitative estimations confirmed this impression. For example, the granules isolated from the pancreas were exceptionally rich in ribonucleic acid, while those from the brain and muscle contained an unusually high amount of phospholipids.

The proteins present in the supernatant fluid after ultracentrifugation gave only weak or negative tests for respiratory enzymes, plasmalogen, and ribonucleic acid. Concerning ribonucleic acid, however, it should be reported that a large portion of this material, which may exceed 50 per cent of the nucleic acid present in the extracts of frog eggs and embryos, as well as of chick embryos, is not sedimented by ultracentrifugation. In the organs of the adult animals, on the other hand, 80 to 90 per cent of the ribonucleic acid extracted from the cells is present in the pellet after ultracentrifugation.

It was already known, from Stern's[39] work, that the particles isolated by ultracentrifugation from heart muscle contained all the cytochrome oxidase, cytochromes, and succinic dehydrogenase which can be extracted with dilute phosphate buffers. The same situation was reported by Chantrenne[15] in yeast cells, where cytochrome oxidase, succinic dehydrogenase, cytochromes a and b, and peroxidase were associated with the granules. Catalase, lactic acid dehydrogenase, carboxylase, and cytochrome c, on the other hand, were present to a much greater extent in the supernatant fluid than in the pellet.

A study of various hydrolases, by Brachet and Jeener,[9] showed that these enzymes are found in the pellet as well as in the supernatant fluid. The enzymes included in this investigation were the following: acid and alkaline phosphatases, ribonuclease, amylase, dipeptidases, cathepsins, trypsin, arginase, and adenylic acid deaminase. Of special interest is the fact that dipeptidases and cathepsins were found in the granules, whatever their origin might have been, and that alkaline phosphatase was so firmly bound to the granules that it could not be removed by repeated washings with buffer solutions. Let us complete the picture by adding that, according to Moog and Steinbach,[33, 40] adenylpyrophosphatase in the chicken embryo is, like the respiratory enzymes of the cytochrome system, present in the granules only.

The next step in our investigation was to find out whether the granules contained the specific proteins elaborated by the various tissues. These studies, due to wartime conditions, were restricted to the pancreas, pituitary, and red blood cells. The results showed that about 60 per cent of the trypsin and at least 20 per cent of the insulin present in the extract of pancreas after low-speed centrifugation was bound to the granules of the pellet. The granules obtained from the hypophysis contained the hormone which produces the expansion of melanophores, while some hemoglobin was always found in the pellet produced from red blood cells. In the latter, it could be shown that this hemoglobin was firmly bound to the granules, since repeated washings failed to remove it.

More recently, Chantrenne[16] has studied the chemical constitution of particles isolated by centrifugation at various speeds. Five different fractions were obtained on centrifugal fractionation of the extract according to the following schedule: Fraction A, 1460 G. for 30 minutes; Fraction B, 5700 G. for 6 minutes; Fraction C, 5700 G. for 60 minutes; Fraction D, 101,000 G. for 6 minutes; and Fraction E, 101,000 G. for 60 minutes. In all five fractions, the content in total nitrogen, ribonucleic acid, lipid phosphorus, alkaline phosphatase, and adenylpyrophosphatase was estimated.

It was found that the smaller particles were relatively richer in ribonucleic acid. The ratio, ribonucleic acid/total nitrogen, increased steadily from Fraction A to Fraction E. In the latter, this ratio was 10 to 15 times larger than in Fraction A. When the content in enzymes and lipid P was related to total nitrogen, it was found that the five fractions, isolated in an arbitrary way, were all different in chemical composition. They cannot possibly be a mixture of only a light and heavy component. This conclusion is supported by observation of the pellets A to E under the ultra-microscope. Each one had its own characteristics; particles in A, B, and C were easy to distinguish, while those in D and E were too small to be seen. These observations led Chantrenne to believe that the granules in the extract were extremely heterogeneous. It is unlikely that they are formed by two entities only, as was assumed by Claude,[20] *e.g.*, by the *large granules* and *microsomes*.

Another interesting point was elucidated by Chantrenne's investigations. If one expresses the results of the analyses in terms of nucleic acid content of the granules, they vary from one fraction to another in a regular way. The larger the particles are, the richer they become in phosphatase, in adenylpyrophosphatase, lipid P, and total nitrogen. As pointed out by Chantrenne, the small granules of Fraction E appear as ribonucleic acid-rich cores around which lipoprotein and enzymes would be synthesized or to which they could attach themselves. Such a hypothesis, in good agreement with Moog and Steinbach's [33, 40] work, is also in agreement with the fact that a large proportion of the ribonucleic acid is not sedimented by high-speed centrifugation of unfertilized frog eggs and early embryos, while a larger proportion can be obtained from tadpole extracts at the same centrifugal speed. It is possible that, during embryogenesis, the small particles become more complex and that this increasing complexity of the granules is an important factor in chemical differentiation.

Pre-existence of the Granules in the Living Cell—Their Physiological Role. It is, of course, of the utmost importance to know whether the granules are already present in the living cell or whether they are only artifacts produced by cytolysis. Careful study of ultracentrifuged pieces of amphibian liver with cytochemical methods brings out strong evidence in favor of the pre-existence, in the living cell, of particles comparable to the granules. After centrifugation of a fragment of frog liver at 40,000 G. for 10 minutes, the basophilic material, which normally is evenly distributed all through the cytoplasm, was thrown down, together with the nucleus, at the centrifugal ends of the cells. These basophilic *crescents* contained ribonucleic acid, as can be readily shown by the ribonuclease test.

We have seen earlier that the isolated granules give positive tests for indophenol oxidase, peroxidase, -SH containing protein, and plasmalogen. All these tests can be used on sections or on small pieces of tissue. It was found that, while they are diffusely positive in the entire cytoplasmic area in the normal liver cells, only the ribonucleoprotein *crescent* stains in the centrifuged liver. It is thus obvious that ribonucleoproteins, -SH containing proteins, plasmalogen, and the respiratory enzymes move together in the ultracentrifuged cell just as they do in the extracts. Since these substances moved towards the centrifugal end of the cell at the same speed used for the isolation of the granules, there is little doubt that they are associated together in the living condition, in a complex identical with or similar to the isolated granules.

This view was further substantiated by an experiment carried out by Chantrenne,[16] at my suggestion, which clearly indicated that cytochrome oxidase was normally bound to granules which play an important role in cellular respiration. Pieces of frog liver were ultracentrifuged at 60,000 G. for four minutes in a solution of saccharose whose density was the same as that of the liver. For purposes of control, non-centrifuged pieces were immersed in the saccharose solution for the same period of time. The oxygen consumption of both fragments was measured and it was seen that ultracentrifugation significantly inhibited respiration.

Further experiments proved that the inhibition involved, essentially, only the cyanide-sensitive respiration which is mediated by cytochrome oxidase. The non-cyanide-sensitive respiratory inhibition obtained by ultracentrifugation was only 6 to 8 per cent, while the cyanide-sensitive respiratory inhibition reached 60 to 70 per cent. These results are similar to those reported by Huff and Boell[29] and by Bodine and Boell[5] for the respiration of *Ascaris* and *Melanoplus* eggs, where ultracentrifugation depressed only the cyanide-sensitive parts of respiration. These observations can be readily explained if one assumes that cytochrome oxidase is bound to granules which ultracentrifugation concentrates in one part of the cell and, accordingly, the cyanide-sensitive respiration in the remaining parts of the cell will necessarily be reduced.

The Possible Role of the Ribonucleoprotein Granules in Protein Synthesis. Any attempt to discuss the role of the ribonucleoprotein granules in protein synthesis is difficult, owing to the scarcity of information on the mechanism of protein synthesis. The most likely possibility (Bergmann and Fruton[2]

and Fruton[25]) is that the action of peptidases and proteases is reversed. The whole problem of protein synthesis by the action of proteolytic enzymes acting on amino acids or peptide mixtures is still in a highly controversial state. There is, however, good evidence, brought forward by Bergmann and his co-workers,[3, 4] that proteolytic enzymes like papain, chymotrypsin, and cathepsin can synthesize a peptide linkage. Such a synthesis, however, is possible only when the synthesized peptides are insoluble. Since the synthesis of peptides is an endo-energetic process, measurable synthesis occurs only when insoluble peptides are formed. Insolubility of the synthesized peptides, of course, displaces the equilibrium of the reaction towards synthesis. It should be pointed out that the physiological dipeptides and tripeptides are not insoluble.

As suggested by Linderstrøm-Lang[30] and by Fruton,[25] it may be that protein synthesis is coupled with exo-energetic processes like oxidation reactions or the breakdown of adenosine triphosphate (ATP) and other compounds that possess energy-rich phosphate bonds.

We cannot, however, entirely dismiss the possibility that other, so far entirely unknown, reactions are responsible for protein synthesis. The situation may be similar to that of glycogen synthesis, which is performed by phosphorylase (Cori[22]), although the action of amylase may also be reversible.

For the time being, we can find out whether the chemical contribution of the nucleoprotein granules agrees in a satisfactory way with the enzyme systems which may conceivably be at work in protein synthesis. It is immediately apparent that such an agreement exists. We always find in the granules the dipeptidases and cathepsin which synthesize peptide linkages under favorable conditions. The granules form a distinct phase in the cell and it is therefore possible that the adsorption of synthesized peptides on such surfaces might shift the equilibria towards synthesis.

Another significant fact is the constant presence in the granules, and only in the granules, of essential respiratory enzymes like cytochrome oxidase and succinic dehydrogenase, as well as of adenylpyrophosphatase. The oxidation reactions and the breakdown of ATP, which have been postulated during protein synthesis, will thus occur in the granules. We saw earlier that the respiratory enzymes, at least the localization in the cell of the granules, is of importance. The fact that the granules always contain some of the specific proteins synthesized by the cells and the progressive complexity of these particles as they become larger constitute additional evidence for their importance in protein synthesis.

The part played by ribonucleic acid itself in protein synthesis is not yet clear. Various suggestions have been made, based mainly on the fact that nucleic acid might combine with the basic groups of amino acids. For example, it is known that nucleic acid combines with many proteins to form insoluble compounds, and it has been suggested by Chantrenne[15, 16] that such a precipitation reaction might be of importance in shifting the equilibrium of the protease reactions towards synthesis.

It seems, however, that a more important lead can be found in Spiegelmann and Kamen's[38] report that there is a flow of phosphorus from the ribo-

nucleoprotein fraction in yeast only when the cells are forced to synthesize proteins. Ribonucleic acid could thus appear as a specific energy donor in protein synthesis as suggested by Spiegelmann and Kamen. It is, of course, a striking fact that ribonucleic acid is among the constant constituents of the granules.

It should, however, be kept in mind that the role of the granules as agents of protein synthesis rests only on circumstantial evidence and not on definite proof. Much more work will be necessary before the exact status of these particles, which have so many characteristics in common with viruses and plasmagenes, can be evaluated.

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—Discussion

Dr. Max M. Friedman (*Queens General Hospital, Jamaica, L. I., N. Y.*): In muscle and heart tissue, the ribose nucleotide content is relatively high and the ribose nucleic acid is low, while in the liver, the reverse occurs. Is there any possible significance to this from the consideration of polymerization equilibrium?

Dr. Brachet: The relationship between ribonucleic acid and ribomononucleotides is still far from clear. There are indications from Ostern's work that, in yeast cells, zymonucleic acid might be transformed into muscle adenylic acid, adenosine triphosphate and, eventually, into coenzymes of the dinucleotide type.

THE SURFACE CHEMICAL PROPERTIES OF CYTOPLASMIC PROTEINS

By M. J. Kopac

Department of Biology, New York University, and Marine Biological Laboratory, Woods Hole, Massachusetts

This report deals with the interfacial denaturation, at oil-water interfaces, of proteins within the living cell as well as those obtained by the centrifugal fractionation of rapidly disintegrated cells. Cytoplasmic proteins undergo little, if any, surface denaturation at experimentally introduced oil-water interfaces providing the cytoplasm remains intact. If the cell is cytolyzed within 30 to 60 seconds after the injection of an oil drop into intact cytoplasm, the surface denaturation of the released proteins is so extensive that the entire interfacial area becomes occupied by protein molecules in various stages of unfolding. The rapid adsorption of proteins and subsequent unfolding causes the interfacial area to expand, thereby producing a crinkled interface—the Devaux effect* (FIGURES 1,A and C, 1,D–G). In the absence of cytolysis, the injected oil drop remains spherical (FIGURE 1,A).

The terms, *surface* or *interfacial denaturation*, as employed in this report, represent the changes in architecture of a globular, 3-dimensional protein molecule to an unfolded, essentially 2-dimensional configuration induced primarily by surface or interfacial forces. The term, *adsorption*, is employed in the classical sense, *i.e.*, concentration of substance at phase boundaries.

The immature eggs of the starfish, *Asterias forbesii*, were employed for the exploratory investigations on the surface chemical properties of cytoplasmic proteins. There were several reasons for this choice of material: (1) the oocytes are large enough so that oil drops can be readily injected into the large germinal vesicle or into the cytoplasm; (2) oil drops may be injected into the cytoplasm without danger of cytolysis, since the cytoplasm of the immature starfish oocyte is remarkably resistant to microsurgical injuries (Chambers[1]); and (3) cytolysis may be readily induced.†

Although the cytoplasm is resistant to injuries produced by careful microinjections of oil drops, the germinal vesicle, by contrast, is extremely susceptible to mechanical injuries. A slight puncture of the germinal vesicle with a microneedle, at room temperatures, is sufficient to initiate rapid cytolysis of the germinal vesicle, nucleolus, and cytoplasm (Chambers[1]).

Thus, one or several oil drops may be safely injected into the cytoplasm. At any given instant, cytolysis may be induced by stabbing the germinal vesicle with a microneedle. The usual micromanipulative procedures as described by Chambers and Kopac[2] were employed.

* Preliminary descriptions of the Devaux effect first appeared in the following: Kopac. Biol. Bull. **71:** 398. 1936; Biol. Bull. **75:** 351. 1938; Micrurgical and Germ Free Methods. pp. 62–76. Thomas, Springfield, Ill. 1943; Colloid Chemistry, vol. 5. pp. 875–883. Reinhold, New York. 1944; Chambers. AAAS Publ. No 14. pp. 20–30. Science Press. 1940.

† At the conference at which this paper was delivered, a motion picture was presented which showed the microinjection of oil drops into the cytoplasm of *Asterias* oocytes, the induction of cytolysis by puncturing the germinal vesicle, the cytolytic reaction, and subsequent development of the Devaux effect at the oil—water interface.

Experimental Modification of Spontaneous Devaux Effects

A. Time of Cytolysis in Relation to Injection of Oil (Kopac[3]).

As shown in FIGURE 1,B, oil drops, after being in contact with intact cytoplasm for several minutes, undergo changes in interfacial properties that prevent the development of the Devaux effect when cytolysis occurs. The effects of various interfacial forces on surface denaturation of proteins will be discussed later.

A series of measurements was made to determine the relation of the time of cytolysis and of the oil injection to the production of Devaux effects. The oil drops were injected at predetermined intervals, either before or after cytolysis. The data are summarized in FIGURE 2.

These data clearly show that rapid, spontaneous Devaux effects developed in nearly every instance when the oils were introduced at or near the onset of cytolysis. If the oil drop remained in contact with cytoplasm for 2+ minutes, about 50 per cent of the drops developed typical Devaux effects. Others crinkled slowly or else the degree of crinkling was moderate (see FIGURES 1,D and 1,E). The critical time for Devaux effect production is therefore about two minutes before cytolysis. Oil drops in contact with cytoplasm for 5+ minutes remained unchanged on cytolysis, as shown in FIGURE 1,B.

On the other hand, if the oils were injected into the cytolyzed residue (post-cytolytic period), the probability of obtaining rapid and intense Devaux effects decreased the longer the interval between cytolysis and injection of the oil. Although all drops crinkled if injected within 15 seconds after cytolysis, only 50 per cent did so when brought in contact with the residue 30 seconds after cytolysis. The spontaneous crinkling diminished in magnitude until, at about 4 or 5 minutes, no visible surface effects were produced. On occasion, oil drops injected 3 minutes after cytolysis slowly developed a mild crinkling (FIGURE 1,D).

The surface denaturation of cytoplasmic proteins during the post-cytolytic period will be further discussed in the section on drop-retraction technique.

B. Effects of Immersion Media on the Production of Devaux Effects.

The *Asterias* oocytes were immersed in various media for periods ranging from 30 minutes to two hours. The percentages of oil drops that developed spontaneous Devaux effects within 45 seconds after cytolysis are given in TABLE 1 (Kopac[4]).

Oocytes immersed in sea water did not always yield the typical Devaux effect if the oil drops were injected two or more minutes before cytolysis (FIGURE 2). On the other hand, oocytes immersed in NaCl or KCl solutions produced spontaneous Devaux effects on oil drops injected up to 4 minutes before cytolysis. NaCl or KCl solutions, obviously, did not produce the same changes at the oil-cytoplasm interface as those observed in oocytes immersed in sea water. The surface denaturation of proteins at oil-water interfaces, as will be discussed later, depends to a considerable degree on the characteristics of the interface.

The action of urea was unexpected. At lower concentrations (0.3 to 0.5M), urea inhibited the production of spontaneous Devaux effects during

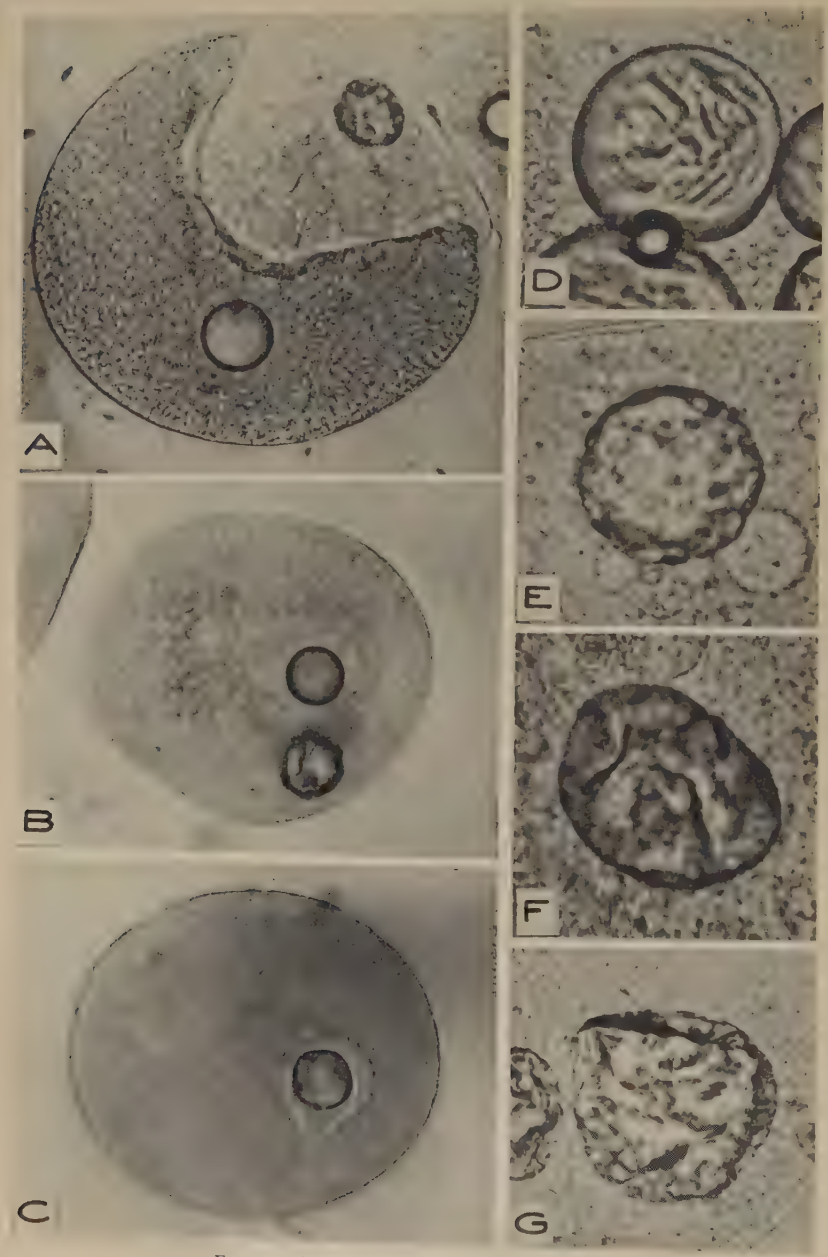

FIGURE 1, A–G. *(For description see facing page.)*

the post-cytolytic periods, although the inhibition on oil drops injected before cytolysis was negligible. At the higher concentration (0.67M), the production of Devaux effects was completely inhibited no matter when the oil was injected into the oocytes. Not only was the Devaux effect suppressed if the oil drops were injected at the instant of cytolysis, normally the optimum time, but there was no suggestion of even mild crinkling. Such oil drops remained permanently spherical.

C. Cytoplasmic Cytolysis Induced by Rapid Microinjection of Aqueous Solutions.

After injecting a drop of oil into the cytoplasm of an *Asterias* oocyte, a second micropipette was employed to inject rapidly a small volume of aqueous solution into the cytoplasm adjacent to the oil drop. Under such conditions, the injection produced a localized cytolytic zone surrounding the oil drop as shown in FIGURE 1,C.

Injections of Salt-free Solutions + Phenol Red. Injections of small volumes of salt-free solutions of phenol red (0.04 per cent) frequently produced small cytolytic zones around only a portion of the oil drop. Accordingly, a portion of the oil drop remained in contact with cytoplasm. The surface of the oil drop, in contact with the cytolytic residue, did not crinkle. The surface, originally in contact with cytoplasm, however, produced a typical Devaux effect after the remaining cytoplasm became cytolyzed.

The phenol red changed in color from red to yellow in the cytolytic zone. Within 30 seconds, the red color reappeared, indicating the infiltration of sea water into the injured zone. Whenever the phenol red in the cytolytic zone remained yellow, no Devaux effects were produced at the oil-water interface.

If a pre-cytolytic zone (narrow zone of cytolytic residue surrounding the oil drop) existed prior to the injection of phenol red, the new cytolytic zone remained red and, in such instances, a mild Devaux effect was developed. This might indicate that the pre-cytolytic zone resulted from the injection of small volumes of sea water simultaneously with the oil. If a pre-cytolytic

FIGURE 1, A–G (*See opposite page*). Photographs of oil drops microinjected into *Asterias* oocytes and the effects of cytolysis.

A. Partially cytolyzed *Asterias* oocyte. *Asterias* oocyte showing an oil drop (Percomorph oil) injected into intact cytoplasm. Note the sphericity and clear-cut contour of oil drop. Another oil drop was injected into germinal vesicle which produced immediate cytolysis. Note the irregular shape and contours of oil mass in cytolytic residue. The change in shape of the oil drop, from a sphere to an irregular mass, occurred in less than 30 seconds. This reaction is called the spontaneous Devaux effect.

B. Oil drops in a cytolyzed *Asterias* oocyte. The first drop was injected into an intact *Asterias* oocyte 8 minutes before cytolysis, the second, one minute before cytolysis. Immediately after cytolysis, the second drop formed a spontaneous Devaux effect within 45 seconds. The first drop remained spherical and did not develop a Devaux effect. Photograph was taken 30 minutes after cytolysis of oocyte.

C. *Asterias* oocyte with small cytolytic zone surrounding oil drop. An oil drop was injected into intact cytoplasm of an *Asterias* oocyte. If small volumes of aqueous substances are rapidly microinjected, localized cytolysis may be induced. Note the clear-cut Devaux effect that has developed at the oil—water interface.

Equilibrium shapes of oil drops following cytolysis of *Asterias* oocytes.

D. The introduction of an oil drop into the cytolytic residue several minutes after cytolysis rarely produces a typical spontaneous Devaux effect. In most instances, only a few crinkles appear at the interface, and these can be demonstrated only if the drop is slightly distorted, for example, by pressing against another oil drop. Note the clear-cut contour of the drop and the spherical shape. Oil drops introduced 5 minutes before cytolysis rarely show more extensive crinkling.

E. Oil drops introduced 1.5 to 2 minutes before cytolysis generally produce a spontaneous Devaux effect of the type illustrated by this photograph. The crinkling is extensive, but the general shape of the drop is spherical.

F. More extensive crinkling produces a considerable distortion in shape of the oil drop.

G. Introduction of appropriate oil-water interfaces at the instant of cytolysis produces the remarkably crinkled and distorted drop shown in this photograph. This is a typical Devaux effect, and the time for maximum crinkling rarely requires more than 30 seconds.

zone was not formed, the Devaux effect failed to appear following the cytolysis induced by phenol red injections.

It is evident that salt-free solutions inhibited the formation of the Devaux effect, but this might be modified either by an alkaline reaction of the cytolytic zone, the entry of sea water, or by the presence of a pre-cytolytic zone.

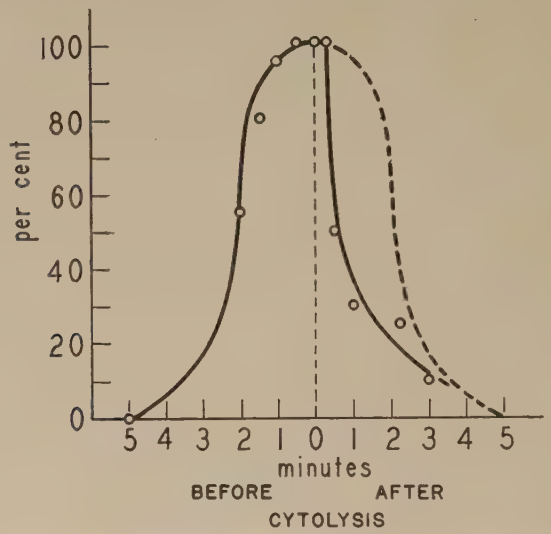

FIGURE 2. The production of spontaneous Devaux effects in relation to time of cytolysis and injection of oil.

The x-axis gives the time in minutes at which the drops were injected either before or after cytolysis. The y-axis gives the percentage of oil drops, at the various injection times, that developed spontaneous Devaux effects within 45 seconds. The extent of crinkling equalled or exceeded that shown by FIGUER 1E.

TABLE 1

Immersion media	*Time of injecting oil in relation to cytolysis*	
	1 minute before	*1 minute after*
Sea water	100 per cent	30 per cent
NaCl 0.52M KCl 0.53M $CaCl_2$ 0.34M $MgCl_2$ 0.37M	90	20
Sea water + urea (0.33 to 0.5M)	90	0
Sea water + urea (0.67M)	0	0

Injections of Potassium Chloride Solutions (0.53M). The oil drops usually developed a moderate Devaux effect (FIGURE 1,E) following cytolysis induced by injections of KCl solutions. In a few isolated instances, typical Devaux effects (FIGURES 1,F and 1,G) were obtained within 30 to 60 seconds.

If a pre-cytolytic zone existed around the oil drop, such residue remained attached to the oil drop following injection of the KCl solution. The residue was hyaline at first, then it quickly disintegrated to a granular residue,

Thus, the KCl solutions had a tendency to inhibit not only the rate but also the intensity of the crinkling reaction at the surfaces of oil drops.

Injections of Calcium Chloride Solutions (0.34M). Spontaneous Devaux effects were produced within 30 to 60 seconds after introduction of the calcium chloride solution. The Devaux effects obtained after cytolysis induced by calcium chloride injections were of the same magnitude regardless of the presence or absence of a pre-cytolytic residue which was usually coagulated. If a pre-cytolytic zone existed, the action of the calcium chloride was to form a shell of coagulated residue around the oil drop.

Oil drops were frequently surrounded by granules following cytolysis by calcium chloride. In most instances, these disappeared and the Devaux effect developed normally. There was no evidence to indicate that these granules were responsible for the Devaux effect. The action of calcium chloride on the Devaux effect was not striking; however, there is a suggestion that the production of the effect was slightly enhanced.

D. Action of Strong Protein Precipitating Agents (Kopac[5]).

The injection of trichloracetic acid into intact *Asterias* oocytes immediately fixed all structures. Within a few seconds, the trichloracetate-treated eggs became so hard that oil could not be injected into them. Injection of trichloracetate into cytolyzed eggs produced an immediate coagulation of the cytolytic residue.

Drops of oil were injected into the cytoplasm of intact oocytes and cytolysis was induced by puncturing the germinal vesicle with a microneedle.

If trichloracetate was injected into the cytolytic residue before the Devaux effect appeared, no further changes at the interface appeared, since the sphericity and smooth contours of the drops were preserved. If trichloracetate was injected at a time when the oil drops were crinkling, no further increase in the Devaux effect occurred.

In every instance, when the precipitant was added after the oil drops had developed the Devaux effect, a reduction occurred in the extent and number of crinkles, *e.g.*, the return to a condition of minimum surface area. Thus, oil drops that were crinkled as shown in FIGURE 1,F before trichloracetate was added generally returned to a shape shown in FIGURE 1,D. These attempts to regain a spherical shape may be due to the shrinking action of the reagent on the adsorbed and on adjacent proteins of the cytolytic residue.

The action of trichloracetate may be summarized as follows:

Oil + cytolysis	Devaux effect [progressive development].
Oil + cytolysis + trichloracetate	Stops development of Devaux effect.
Devaux effect + trichloracetate	Reversal, to spherical shape.

The injection of an aqueous solution of phosphotungstic acid into cytolyzed oocytes produced an immediate coagulation. Frequently, on cytolysis there may be an outflow of cytolytic residue through a torn portion of the vitelline membrane (FIGURE 9,A). Injection of phosphotungstate into such oocytes immediately stopped the outflow of such cytolytic residue. The action of the reagent was localized, and small amounts of it produced only small coagulation zones.

With oil drops injected into intact oocytes and cytolysis produced in the usual manner, the effects of phosphotungstate were to stop further development of the Devaux effect. As soon as the precipitant came in contact with the region surrounding the oil drop, no further changes occurred either in increasing the number of folds or in decreasing the extent of crinkling (see trichloracetate effect). Even after prolonged standing (12 to 24 hours), there was no tendency of the distorted drops to return to spherical shapes.

Accordingly, the action of phosphotungstate may be summarized as follows:

Oil + cytolysis	Devaux effect [progressive development].
Oil + cytolysis + phosphotungstate	Stops further development of Devaux effect.
Devaux effect + phosphotungstate	No reversal.

To summarize, the action of either trichloracetate or of phosphotungstate was to prevent further development of the Devaux effect. Trichloracetate reduced the interfacial area of the crinkled drop, while phosphotungstate left the crinkled interface unchanged. In both instances, the most striking effect was the immediate stoppage of any further increase in crinkling, folding, or distortion of the oil drops. When the precipitants were added before crinkling had started, such drops retained their original sphericity and smooth contours. In other words, these reagents inhibited the surface denaturation of cytoplasmic proteins.

E. Devaux Effect in Centrifuged Oocytes and Mature Eggs (Kopac[6]).

Centrifugal technique was employed for separating the formed components within the cells. *Asterias* eggs, immature or mature, may be centrifuged for 10 to 12 minutes at 6 to 7 kilogravities without serious injury, providing the cells are suspended in an isopycnotic mixture of sucrose and sea water. Following such centrifugation, the formed components of mature eggs were separated into three zones: (1) an oil cap, composed of oil globules, at the extreme centripetal pole; (2) a large granular layer at the centrifugal pole; and (3) a narrow, hyaline band consisting of cytoplasmic matrix, between the oil cap and the granular zone (see also Costello[7]).

A hyaline zone did not appear in the cytoplasm of centrifuged, immature eggs, since the centripetal pole was occupied by the persisting germinal vesicle. It is of interest to note that the nucleolus is the densest structure within the germinal vesicle, since its location after centrifugation is always in the extreme centrifugal zone of the vesicle.

Hyaline versus *Granular Zones in Centrifuged Eggs.* Oil drops were injected into the hyaline and into the granular zones of centrifuged, mature eggs. The cell was induced to disintegrate by repeated prodding with a microneedle. The rate and extent of development of the Devaux effect was recorded for both oil drops.

The differences in the degree of development of the Devaux effect on the two drops were indeed striking. The oil drop in the centrifugal, granular zone quickly became distorted and covered with numerous folds (FIGURE 1,G), while the oil drop in the hyaline zone either formed a few surface

crinkles (FIGURE 1,D) or remained unchanged. The rates of development were considerably different. The Devaux effect was developed on the oil drop in the granular zone within a few seconds, while in the hyaline zone, if changes occurred, a considerable period of time elapsed before these changes became apparent. In no instance did an oil drop, placed in the hyaline zone, attain the degree of crinkling shown, for example, by FIGURE 1,E.

The typical experiment, following cytolysis, would show a heavily crinkled oil drop near the centrifugal end and a spherical, relatively unchanged oil drop near the centripetal end. The average time for oil drops, placed in the centrifugal, granular zone, to develop a maximum Devaux effect was about 15 seconds, and several instances were noted where such changes occurred in less than 7 seconds.

Another significant point was that complete cytolysis of the granular zone was unnecessary. The oil drop needed only to be surrounded by a small cytolytic zone to produce the striking, spontaneous Devaux effect.

Centrifuged, Immature Oocytes. Centrifuged oocytes produced different reactions from those observed in similarly treated, mature eggs. Employing the same procedures as outlined above, an intense and rapid Devaux effect was produced in both the germinal vesicular zone and in the granular zone. The rates and intensities were essentially the same. The average time for complete crinkling (FIGURE 1,G), considering all instances, ranged from 15 to 40 seconds.

Since no visible granules were present in the germinal vesicle, the results obtained with the oil drops are of considerable interest. It is obvious that the hyaline substance within the germinal vesicle differs from the hyaline zone of the centrifuged, mature egg. Thus, by use of the Devaux effect as a criterion, the two hyaline-appearing substances could be as clearly differentiated as by the use of ultra-violet light absorption (see Harvey and Lavin[8] regarding the *Arbacia* oocyte).

In centrifuged, mature eggs, an intense Devaux effect occasionally developed in the hyaline zone. However, this was due to the disintegration of the membrane of the germinal vesicle following centrifugation. In such examples, the contents originally present in the germinal vesicle were still confined to the apparent hyaline zone of the matrix. Thus, if the germinal vesicular membrane was allowed to break down, following centrifugation, before the oil drops were injected, rapid and intense Devaux effects could be demonstrated on subsequent injection of oil drops.

In other instances, although the Devaux effect did occur in the apparent hyaline zone, the effect was slower and less intense (FIGURES 1,E and 1,F) than in the previous examples. Such an effect might have resulted from the disintegration of the membrane during centrifugation and the diffusion of the contents of the germinal vesicle into the surrounding cytoplasm. The inevitable redistribution of cellular inclusions, following centrifugation, produced a dilution of material from the germinal vesicle which is responsible for the Devaux effect in immature eggs. Only in those cells which maturated prior to centrifugation was the Devaux effect negligible in the hyaline zone.

It is reasonable to suppose that the substances in the germinal vesicle which become dispersed in the cytoplasm following maturation may be carried by the dense granules during centrifugation. This would tend to deplete the hyaline zone of those substances that promote the development of spontaneous Devaux effects.

Comparison of the Rates of Devaux Effect Development in Centrifuged and in Uncentrifuged Eggs. The role of the granular components of the *Asterias* egg in producing the Devaux effect on oil drops was analyzed in another way. A tabulation was made of the times required for complete crinkling on oil drops injected into uncentrifuged, mature eggs or into the granular zones of centrifuged eggs.

The results are summarized in TABLE 2.[5]

The times given in TABLE 2 are the intervals from the instant of cytolysis to completion of the Devaux effect. Only those drops on which crinkling occurred were considered. In the uncentrifuged cells, 4 out of 112 (ca. 4 per cent) failed to reach the stage illustrated by FIGURE 1,G within 45 seconds, but the crinkling was completed within 50 to 60 seconds.

TABLE 2

Per cent of oil drops showing maximum Devaux effect	*Time in seconds for development of Devaux effect*		
	Centrifuged, granular zone.	*Uncentrifuged, cytoplasm.*	$\frac{\text{Uncentrifuged}}{\text{Centrifuged}}$
70	17	30	1.76
80	18	32	1.78
90	20	36	1.80
100	30	45	1.50

The above data show that the granular components are responsible for the increased crinkling rates in mature eggs. The granules are concentrated by centrifugation and, accordingly, the degree of crinkling seems to be a function of the concentration of the adsorbable material.*

Disintegration of Granules Stained with Methylene Violet. *Asterias* eggs readily accumulated the thiazine dye, methylene violet (Bernthsen), from dilute solutions in sea water. The same granules that could be stratified centrifugally were stained blue. Under low power, the uncentrifuged eggs appeared to be uniformly stained, but this was due to the large number and regular distribution of the granules. The nucleolus also became stained, but more slowly than the granules.

Striking changes in coloration of the cytoplasm of uncentrifuged eggs occurred after cytolysis. On cytolysis, the stained granules disintegrated. Where a large cytolytic zone was produced, the cytoplasmic residue became

* According to Costello,[7] the packed volume of granules in *Asterias* eggs is ca. 77 per cent of the total egg volume. On correcting this volume for packing and subtracting the volume occupied by intergranular matrix, the volume becomes ca. 57 per cent. Thus, on centrifugation, the concentration of the granules becomes 0.74 of the total volume occupied. Originally, these granules occupied 0.57 of the egg volume. The ratio, 0.74/0.57 = 1.34, or a 34 per cent increase in granular concentration. The increase in rates of Devaux effect development on centrifugation ranges from 50 to 80 per cent (TABLE 2). These values agree surprisingly well with those calculated from Costello's data.

faintly colored, while the granules in the intact cytoplasm retained their blue color.

Following the injection of oil drops into the cytoplasm of stained eggs, and on inducing cytolysis only around the oil drop, the effects most frequently observed were the following: A typical spontaneous Devaux effect was developed on the oil drop (FIGURE 1,G). The granules disintegrated in the cytolyzed zone, which became either colorless or faint blue. In many instances, the surface of the oil drop acquired a blue color. As long as the granules remained intact, the color was retained by them.

Stained, centrifuged eggs gave results that were identical to those obtained with unstained cells. On cytolysis, the stained granules disintegrated and the oil drops became crinkled. Oil drops placed into the hyaline zone behaved similarly to those placed in the hyaline zone of unstained eggs. If the stained granules were present in small amounts, the Devaux effects were moderate (FIGURE 1,E). Maximum effects were obtained where the granules were the most numerous, *i.e.*, in the centrifugal zone.

In stained, immature eggs which were also centrifuged, the Devaux effects were obtained in the germinal vesicular zone even though there was no accumulation of the dye, since the granules were sparse. At best, the concentration of stained granules in the region of the germinal vesicle of strongly centrifuged eggs was low. The differentiation of the germinal vesicular contents from the hyaline cytoplasmic matrix was again demonstrated.

Disintegration of Granules Stained by Rhodamine. The granules of *Asterias* eggs were stained a deep pink color while the hyaline matrix was only faintly stained in cells immersed in dilute solutions of rhodamine in sea water.* On cytolysis, the granules disintegrated as usual, releasing the dye, which in turn stained the non-granular components a deeper pink color. The Devaux effects were identical to those obtained in unstained eggs or in eggs stained with methylene violet.

Even when the cytolysis was confined to the region surrounding the oil drop, the intact cytoplasm as well as the cytolyzed zone was stained. The latter effect, therefore, is different from that obtained under similar conditions in eggs stained with methylene violet.

It is evident, therefore, that the Devaux effects obtained at oil-water interfaces placed in contact with the cytoplasm of *Asterias* eggs depends on the breakdown of those granules which accumulate methylene violet or Rhodamine. These dyes had no effect on either the rate or extent of Devaux-effect development. The rates and intensities of crinkling at oil-water interfaces, however, were increased in accordance with the concentration of the granules.

The Devaux effects, on the other hand, that were obtained from the germinal vesicle or contents of this structure were independent of granular disintegration. Such effects resulted from the adsorption and subsequent surface denaturation of hyaline components present in the non-granular structure of the germinal vesicle.

* E. B. Harvey (Biol. Bull. **81**: 114. 1941) reported that the yolk granules and mitochondria of *Arbacia* eggs are stained a pink color by rhodamine. The upper portion of the matrix fraction also stained a pink color with rhodamine.

The most significant observations were that Devaux effects could not be obtained unless a significant amount of cytolysis occurred around the oil drop, regardless of the nature of the oil-water interface. While the cytoplasmic or the germinal vesicular structures remained intact, the injected oil drops remained spherical as shown in FIGURE 1,A.

The Drop-Retraction Technique

The striking differences between cytoplasmic proteins, *in vivo*, and those obtained after cytolysis, as demonstrated by surface chemical behavior, required an investigation of the interfacial denaturation of simpler proteins and the effects of various agents on these properties.

One of the most useful methods to be developed for these determinations has been the drop-retraction technique and the micro-tensiometer (Kopac[9, 10]). These techniques are micro-adaptations of surface chemical procedures, originally developed by Devaux,[11] and extended by Langmuir and Waugh,[12] for the investigations of protein films at oil-water interfaces.

The apparatus permits the measurement of surface denaturation of proteins at oil-water interfaces in which the oil drops are of the same dimensions as those shown in demonstrating spontaneous Devaux effects (FIGURE 1,A to G) and post-cytolytic phenomena (FIGURE 9,A and B).

Several new features have been added to the apparatus and to the procedures since the drop-retraction technique was first described (Kopac[10]). These will be discussed and to this will be added representative data on model protein and nucleoprotein systems.

With the drop-retraction apparatus, the surface area of an oil drop, poised on the tip of a micropipette, can be increased or decreased by enlarging or reducing its diameter. FIGURE 5 is a pictograph illustrating the technique and FIGURE 8 illustrates the apparatus with which the dimensions of an oil drop may be precisely controlled and measured.

The micropipettes were made by hand in such a way that during the pulling-out process an orifice was formed and fire-polished. As described by Barber,[13] pipettes with a small polished opening are obtained when the glass, in a half-molten state, parts with a gentle snap. To produce such pipettes, one must pull suddenly when the glass begins to melt in the microflame. The diameters of the orifices ranged from 2.5 to 10 microns. Since the orifice is fire-polished and at right angles to the axis of the pipette, the oil drop remains poised on the tip of the pipette as shown in FIGURE 4,A.

The manual control of the drop-retractor (FIGURE 5) is employed to charge the micropipette with a volume of oil by filling through the tip from a small hanging drop of oil previously placed on a coverslip and mounted on the moist chamber. With the micropipette filled with oil, and the aqueous phase in place, the tip of the micropipette is inserted into the hanging drop. The pressure necessary to expel the oil can be developed by manipulating the micrometer head. If not too large, the spherical drop will remain attached to the microtip as shown in FIGURE 4,A. If too large, it will break away, and the pipette must be refilled with a smaller volume of oil.

Factors which limit the maximum size of an oil drop that will remain

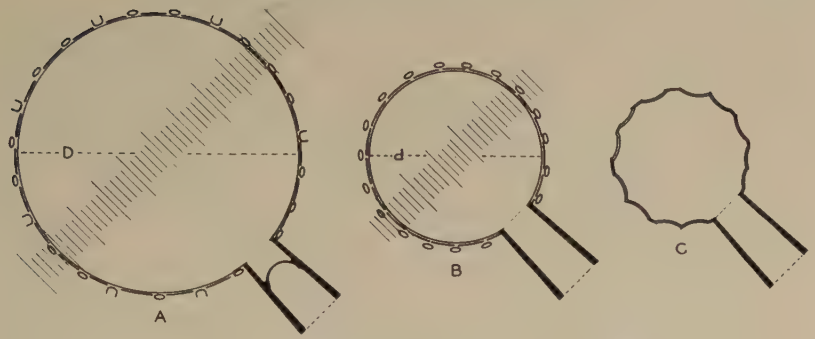

FIGURE 3. Diagrams of oil drops at various stages of retraction. These diagrams, representing cross sections of oil drops attached to the micropipette, explain the crinkling effect.

A. In an aqueous phase containing proteins, an oil-water interface, at any time, will contain protein molecules in various stages of unfolding. First, some molecules remain as 'native,' 3-dimensional structures (ellipsoids). Second, other molecules will be partially unfolded (U-shaped). Third, still others will be completely unfolded (2-dimensional platelets).

B. The interfacial area becomes reduced on retracing the oil drop. Molecules present in the interfacial zone are subjected to increasing surface pressures as the interfacial area is reduced. As the surface pressure is increased, some of the molecules present in *A* will be squeezed out of the interface. The first molecules to be expelled will be the 3-dimensional molecules since they have undergone no changes in structure and, accordingly, their original solubility in the aqueous phase is preserved. Further decrease in interfacial area with accompanying increase in surface pressure will expel the partially unfolded molecules. The surface pressure needed to force out the partially unfolded molecules may depend on the degree of unfolding. It is possible that partially unfolded molecules cannot be completely expelled, but only the less unfolded portions will be pushed out of the interfacial area. The fully unfolded molecules cannot be driven out of the interface no matter how high the surface pressure. These molecules have oil on one side and water on the other. This figure represents the condition of the interface at the critical diameter, *d* (see also FIGURE 6).

C. If the interfacial area is further reduced, crinkling will result, since the interfacial area cannot be made any smaller due to the presence of the nondisplaceable, unfolded molecules. Reduction of the volume of the enclosed mass while interfacial area remains constant produces the crinkling effect.

The diameters, *D* and *d*, of the oil drops can be measured with a calibrated micrometer ocular. The drops may also be photographed (FIGURES 6 and 8) and the measurements obtained later from negatives.

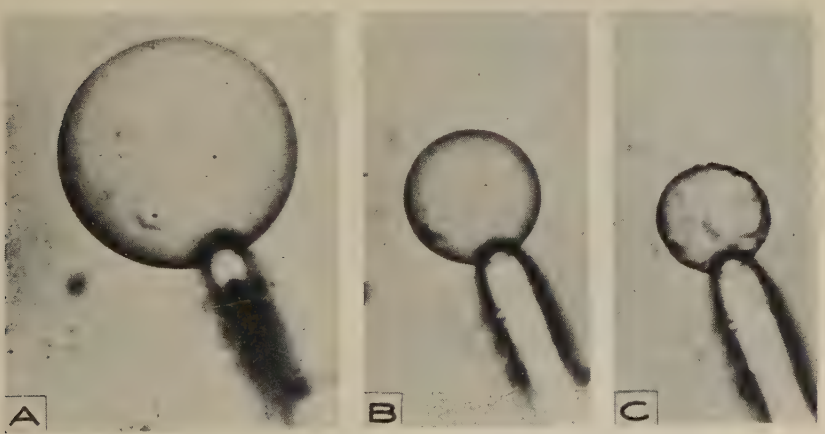

FIGURE 4. Photographs of oil drops in contact with an aqueous phase containing proteins.

Practical application of the drop-retraction method. Note the small volume of oil remaining in the tip of the micropipette. With this oil plug, the oil may be drawn back into the pipette by reducing the back pressure with the drop-retractor (FIGURE 7).

The surface area of the first drop, $A = \pi D^2$, where D is its diameter. With the oil phase immersed in clean water or in inorganic salt solutions, the drop maintains a spherical shape until it is completely retracted. If, however, the oil first comes in contact with an aqueous phase containing proteins, the retraction of the oil produces strikingly different effects. As the drop (B) is retracted, by reducing the back pressure, the sphericity of the drop will be maintained to the critical diameter, d. Its surface area, $s = \pi d^2$.

If the drop (C) is made any smaller, the surface becomes crinkled, thereby simulating the spontaneous Devaŭx effect.

attached to the micropipette are the following: (1) the weight of the oil drop ($= V \Delta d\ g$), where V = volume of oil drop (cc), Δd = difference in density between oil and aqueous phase, g = acceleration by gravity (980 cm/sec^2), and (2) the surface forces holding the oil drop to the pipette ($= 2r\pi T$). Here, r = radius of the orifice (cm) and T = oil-water interfacial tension (dynes/cm).

If $V \Delta d\ g > 2r\pi T$, the drop will fall away. Accordingly, for the drop to remain attached, the following relationship must be maintained: $V \Delta d\ g < 2r\pi T$.

Experimental observations have shown that micropipettes with large orifices can maintain larger drops than micropipettes with smaller openings at the microtip. Moreover, oils with higher oil-water interfacial tensions can form larger, stable drops than oils with low values of T.

With the oil drop immersed in clean water or in inorganic salt solutions, the drop maintains a spherical shape until it is completely retracted following the application of a negative pressure with the drop-retractor (FIGURE 5). A strikingly different situation will develop if the oil first comes in contact with an aqueous phase containing proteins. As the drop is retracted by reducing the back pressure, the sphericity of the drop can be maintained up to a certain degree, the critical diameter (FIGURES 4,B and 6,A). If the drop is made any smaller, the surface becomes crinkled (FIGURES 4,C and 6,B), thereby producing the Devaux effect. The crinkling can be explained by referring to FIGURE 3.

If the retraction is done properly, none of the protein molecules adsorbed at the interface (FIGURE 3,A) can enter the pipette with the oil since the lip of the micropipette serves as a barrier. This arrangement is analogous to the straight-edge barrier used in a conventional Langmuir film trough. The method is essentially a micro-adaptation of the procedure originally developed by Devaux[11] for measuring film characteristics of protein monolayers at liquid-liquid interfaces. In Devaux's method, the area of the interface was expanded or reduced by raising or lowering the water level in the bulbous portion of a flask.

FIGURE 5 (*See opposite page.*)

A moist chamber, mounted on the stage of a microscope, supports a coverglass from which is suspended a small volume (20 to 40 cmm.) of aqueous phase in the form of a hanging drop. The moist chamber, by avoiding evaporation or condensation, maintains the concentration of the components in the aqueous phase at a constant level for considerable periods.

The micropipette and its holder are mounted on the left side of the Chambers' micromanipulator. With this instrument, the micropipette can be moved with precision to any region within the microscopic field.

A fine-bore, flexible copper tube joins the micropipette to the drop-retractor. The metal tube, pipette holder, and syringes (on drop-retractor) are filled with distilled water. The volume within the shaftlet and the shaft of the micropipette is filled with air, thereby providing a small compressible volume within the system.

The diameter of an oil drop, poised on the tip of the micropipette, can be precisely controlled by the drop-retractor, an instrument consisting of the following parts: (1) manual control, a 2 ml Luer syringe activated by a micrometer head, (2) semi automatic control, a 5 ml Luer syringe activated by a slow-speed, motor-driven feed screw, (3) stopcocks and reservoir for filling syringes and connecting tubes with water, and (4) gauge for indicating pressure in the micropipette.

The small gas volume within the shaft of the micropipette can be compressed or expanded by appropriate movements either of the micrometer head or of the motor-driven feed screw. Negative pressure will cause the oil to flow into the pipette and positive pressure will drive it out again. Both the micrometer head and motor-driven feed screw are sufficiently fine so that the diameter of the oil drop can be reduced in steps of less than 1 micron.

The manual control is employed in filling the micropipette, through the tip, with oil from a hanging drop of oil mounted on the moist chamber. This volume will make up the oil drop less the small residuum (FIGURE 6) that must be left in the microtip so that the oil mass may be subsequently retracted.

With the pipette charged with oil and the aqueous phase in place, the tip of the micropipette is inserted into the hanging drop. Pressure is built-up by manipulating the micrometer head to expel the oil from the micropipette. The semi-automatic control is used to retract the oil drop. The motor is operated either with a foot switch or by the electric timer (FIGURE 8).

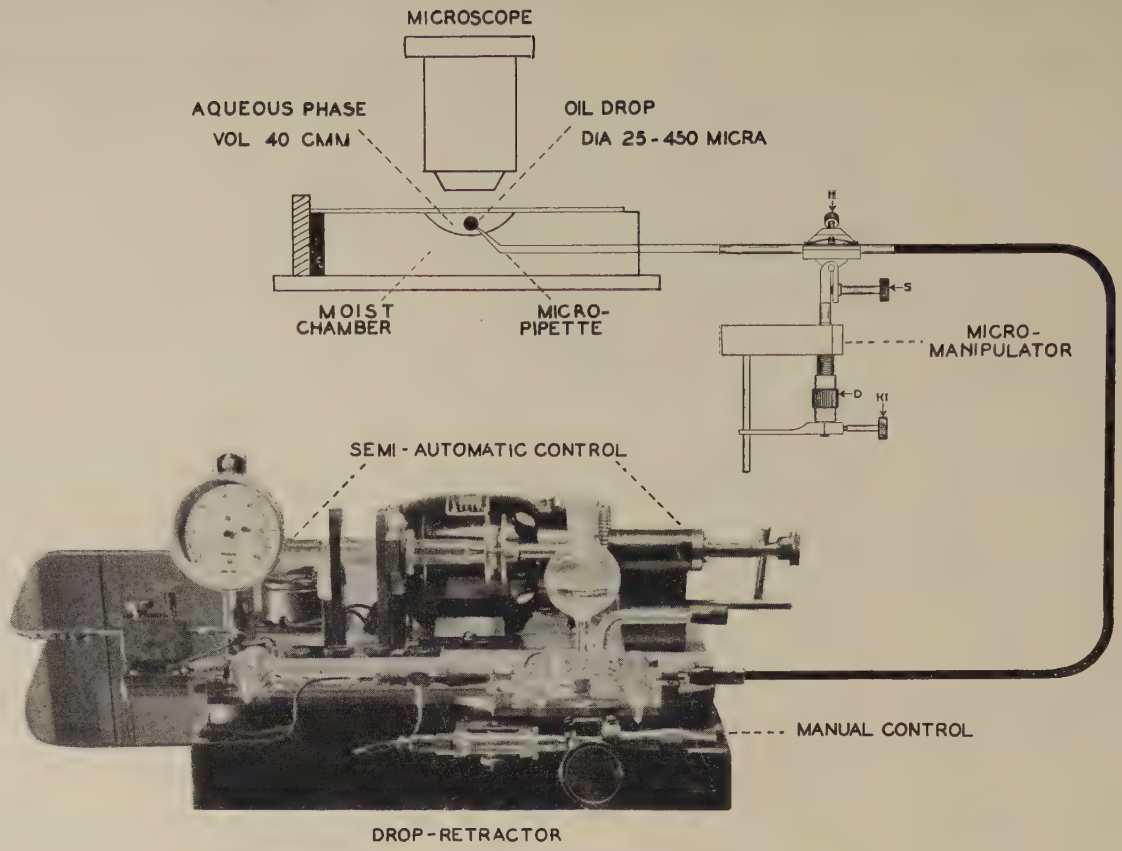

FIGURE 5. Pictograph of the drop-retraction apparatus. *(For description see facing page)*

An oil drop of diameter, D, in contact with the aqueous phase presents an interfacial area at which protein molecules may undergo surface denaturation (FIGURE 3,A). This oil drop, at any time, can be retracted to the point that crinkling would occur providing the drop were further reduced in size. On reducing the drop slightly below the critical diameter (FIGURE 6,A), the drop shifts in position indicating a tendency of the drop to fall away from the pipette.

Before retraction, $V_o \Delta d\ g < 2r\pi T_o$ (V_o = volume of drop of diameter, D, and T_o = oil-water interfacial tension), but, as T is diminished by the interfacial concentration of protein molecules, the following situation arises: $V_t \Delta d\ g > 2r\pi T_t$ (T_t = oil-water interfacial tension at time of retraction,

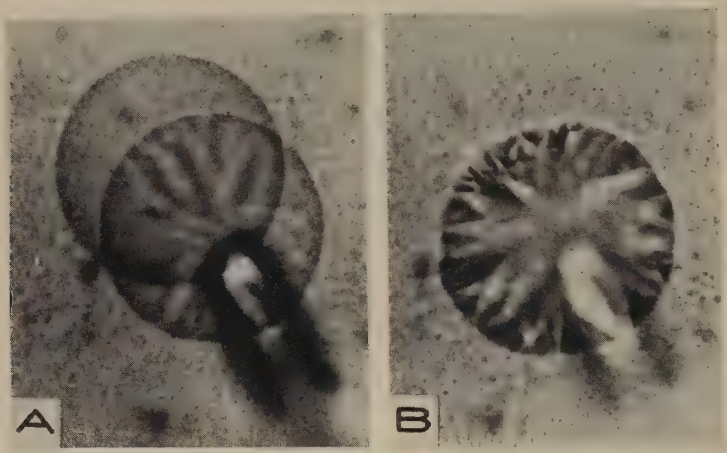

FIGURE 6. Photographs of oil drops in contact with an aqueous phase containing cytoplasmic proteins and granules isolated from sea-urchin eggs.

The intentional double exposure (A) shows the smallest diameter of an oil drop which, under a given experimental condition, remains spherical, and the appearance of the drop when the critical diameter is slightly exceeded. Note that the spherical drop is poised on the end of the micropipette. This represents the critical diameter, d.

On reducing the drop slightly below the critical diameter, the drop shifts in position. In all instances, the drop quivers at the instant the critical diameter is reached, thus providing an index so that the exact critical diameter may be measured.

Further retraction of the oil mass results in the crinkled surface shown in B. The interfacial film is rigid. While the crinkled state is maintained, it is difficult to separate the oil mass from the micropipette. These photographs illustrate the principle of the Devaux effect, *e.g.*, the crinkling is produced by a spontaneous expansion of the interface.

and V_t = volume of oil drop at the same time). This situation is indicated by the appearance of instability and is shown by displacement in position of oil drop (FIGURE 6,A). The diameter of the oil drop at the instant of quivering is taken as the critical diameter. The drop quivers at the instant the critical diameter is reached, thus providing an index so that the exact critical diameter, d, can be measured.

The critical interfacial area, $s = \pi d^2$, represents the total area of protein molecules that cannot be driven out of the oil-water interface by application of surface pressures ranging from 20 to 50 dynes/cm.* The magnitude of the

* The drop-retraction method produces a surface compression of the fixed barrier type. The spreading force, F, is approximately equivalent to the following: $F = T_o - T_x = nkt/(\pi d^2 - nA)$. Here, T_x and T_o, are the interfacial tensions of the oil-water interface, with and without protein molecules; k = Boltzmann's constant; t = absolute temperature; πd^2 = surface area of oil drop of diameter, d; and nA = area occupied by n molecules at the interface.

surface forces depends mainly on the components of the oil-water interfacial system. The interfacial area, s, at any time, includes protein molecules that may be completely unfolded and also portions of molecules that cannot be expelled from the interface, although these molecules may not be entirely unfolded.

The maximum area available for non-displaceable protein molecules is represented by $A = \pi D^2$. Accordingly, a convenient means of indicating the extent of surface denaturation of protein molecules is by the fraction, s/A. This fraction is evaluated by calculating the ratio, $\pi d^2/\pi D^2$.

The values of s/A, at any time, give an estimate of the area of unfolded or surface denatured molecules per unit area of interfacial surface. The value of s/A may also include portions of the partially unfolded molecules. Succeeding values of s/A will include those molecules present during preceding measurements plus those that have become unfolded since then. In most instances, molecules that have become completely unfolded represent those that were only partially unfolded during previous measurements. The changes of s/A with time depend on the oil-water interface, the type of proteins, pH and ionic strength of the aqueous phase, and other factors.

The equations relating interfacial denaturation to time become:

$$s/A = kt^a, \text{ or } log\ s/A = log\ k + a\ log\ t \text{ (FIGURE 7)}.$$

At $t = 1$, $log\ s/A = log\ k$, while $a =$ the slope, or $tan\ \Theta$, of the $log\ s/A$ versus $log\ t$ curve. All values can be obtained from the logarithmic graph, and one may, therefore, use the values of k and of the slope, a, in comparing the surface denaturation of proteins under various interfacial conditions.

Preliminary results (Kopac[5]) from a new series of measurements suggest that a major fraction of the increase in s/A is due to the progressive unfolding of protein molecules at the interface. Prior to these recent measurements, the increase in s/A was ascribed to either one of two possibilities: (1) increase in number of protein molecules unfolding at the interface or (2) increase in unfolding of molecules initially present at the interface.

The second possibility now appears to be more probable since the changes in s/A on oil drops left in media containing bovine plasma albumin molecules were not appreciably different on transferring oil drops to an albumin-free medium. These data suggest that an increase in number of molecules capable of unfolding at the interface is not especially significant since, on transfer to the protein-free medium, the probability of increasing the number of such molecules is drastically reduced. It would appear, therefore, that the increase in s/A must result from the further unfolding of those molecules acquired by the interface during the early sojourn of the oil drop in an albumin-containing medium.

In some instances, k may actually represent the initial adsorption and preliminary unfolding of proteins present at the interface, while the slope, a, is a measure of the rate of unfolding of those molecules initially adsorbed.

The protein molecules present, at certain oil-water interfaces, may unfold enough during the first minute so that they cannot be entirely displaced on drop-retraction. The unfolded portion of these molecules would remain

in the interface on drop-retraction while less unfolded portions might be forced out. This quantity would be expressed by k.

On re-expansion of the oil drop, the displaced portions would reoccupy interfacial space, and thereby prevent new molecules from entering the interface. With time, more of each molecule would become non-displaceable on drop-retraction. This would be shown by the slope, a.

Where k is high, the slope, a, may not be a true indication of the rate of unfolding, owing to space difficulties involved in subsequent unfolding of the molecules when the interface becomes crowded.

With low k and high a values, the situation may be complex. Thus k is undoubtedly an indication that both the number of molecules and their

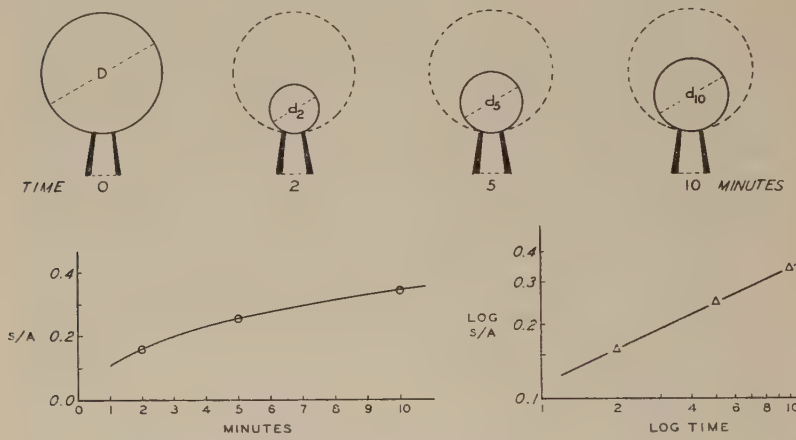

FIGURE 7. Quantitative aspects of the drop-retraction method.

As soon as the oil drop was brought in contact with the aqueous phase, the time was set at zero and its diameter, D, measured. At 2 minutes, the drop was retracted to its critical diameter, d_2, and measured. Immediately thereafter, the drop was expanded to its original diameter, D. At 5 minutes, the drop was again retracted to its new critical diameter, d_5, measured, and re-expanded to diameter, D. At 10 minutes, the new critical diameter, d_{10}, was measured, and so on. If subsequent measurements are to be taken, the drop must be re-expanded to its original diameter, D. Usually, these measurements are terminated after 15 minutes but some have been continued for periods as long as 90 minutes.

The values of s/A are calculated for each value of d as measured at the various times, since $s/A = \pi d^2/\pi D^2$.

If one plots the values of s/A against *time*, a parabolic curve is obtained as shown in the left graph. A straight line is obtained on plotting *log* s/A against *log* t (right graph).

degree of unfolding is low. The subsequent high value for the slope, a, may be accounted for in one of 3 ways: (1) an increase in number of molecules that become adsorbed with time, (2) increase in the degree of unfolding of those molecules present at $t = 1$, and those acquired later, or (3) a combination of the first two.

The crinkled state is developed on further compression of the interface by reducing drop diameter (FIGURES 4,C and 6,B). It is not difficult to understand that a point can be reached where the interface becomes completely packed with unfolded and trapped protein molecules (FIGURE 3,B). The unfolded molecules are so oriented that the hydrophobic surfaces (non-polar amino acid residues) are directed towards the organic phase, while the hydrophilic surfaces (polar amino acid residues) are directed towards the aqueous phase. Such molecules become trapped at the phase boundary

since they are no longer soluble in the aqueous phase, nor are they soluble in the oil phase. Further reduction of the interfacial area becomes impossible and any attempt to do so results in the crinkling effect (FIGURES 3,C and 6,B).

Since drop-retraction increases the interfacial concentration of surface denatured proteins, the value of T will be lowered thereby (Kopac[10]). As the critical diameter is approached, the surface concentration of unfolded proteins increases and T becomes correspondingly reduced and may approach zero. This situation is indicated by the appearance of instability, *i.e.*, quivering (FIGURE 6,A).

Failure of the drop to fall away, at this time, is a result of the elastic nature or yield value of the surface denatured proteins which, when concentrated and compressed, form a membrane of appreciable mechanical strength (FIGURE 6,B).

While the crinkled state is maintained, it is difficult to separate the oil mass from the tip of the micropipette. These data indicate that proteins not only become surface denatured but, on compression, they may also become surface coagulated. The yield values of such protein films are high and compensate remarkably for the low (real) interfacial tension that is developed when the critical diameter is reached or exceeded.

The quantitative aspects of the drop-retraction technique are explained in FIGURE 7. The values of s/A may cover a range from 0 to 1.0. If $s/A = 0$, no surface denaturation has occurred; if $s/A = 1.0$, the interfacial area is entirely occupied by non-displaceable, surface denatured protein molecules.

The Devaux effect that develops following cytolysis of a cell at appropriate oil-water interfaces is produced by the interfacial adsorption and denaturation of protein molecules. Under such conditions, the value of $s/A = 1.0$, and this value is reached spontaneously. Where protein adsorption and unfolding is less pronounced, the spontaneous Devaux effect does not form, and the degree of surface denaturation may be estimated only by the application of the drop-retraction procedure as shown in FIGURES 5 and 7.

Application of the Drop-Retraction Technique to Cells

As previously stated, the longer the time interval between cytolysis and application of the oil, the lower is the probability of obtaining spontaneous Devaux effects (FIGURE 2). In fact, after a few minutes have elapsed, only a slight crinkling may develop at the interface (FIGURE 1,D), and, after about 5 minutes, no changes on the surface of the oil may be observed. The question remains whether the substances that ordinarily produce the Devaux effect disappear completely or whether the concentration diminishes with time. To determine satisfactory conclusions on this point, the drop-retraction method was employed, since the technique is sufficiently refined so that it can be used within the dimensions of a single cell.

In FIGURE 9,A the micropipette can be seen with an attached oil drop placed near the margin of an *Asterias* oocyte introduced about 5 minutes after cytolysis. After 10 minutes' exposure to the cytolyzed residue, the drop

was retracted until a partial Devaux effect was obtained. In this example, the value of s/A, as calculated from the two surface areas (FIGURE 7) was *ca.* 0.9. This value indicates that a considerable amount of protein was surface denatured at the oil-water interface, but this amount was insufficient to produce a spontaneous Devaux effect ($s/A = 1.0$).

In general, the longer the period between cytolysis and application of the oil, the smaller will be the value of s/A as calculated from the measured

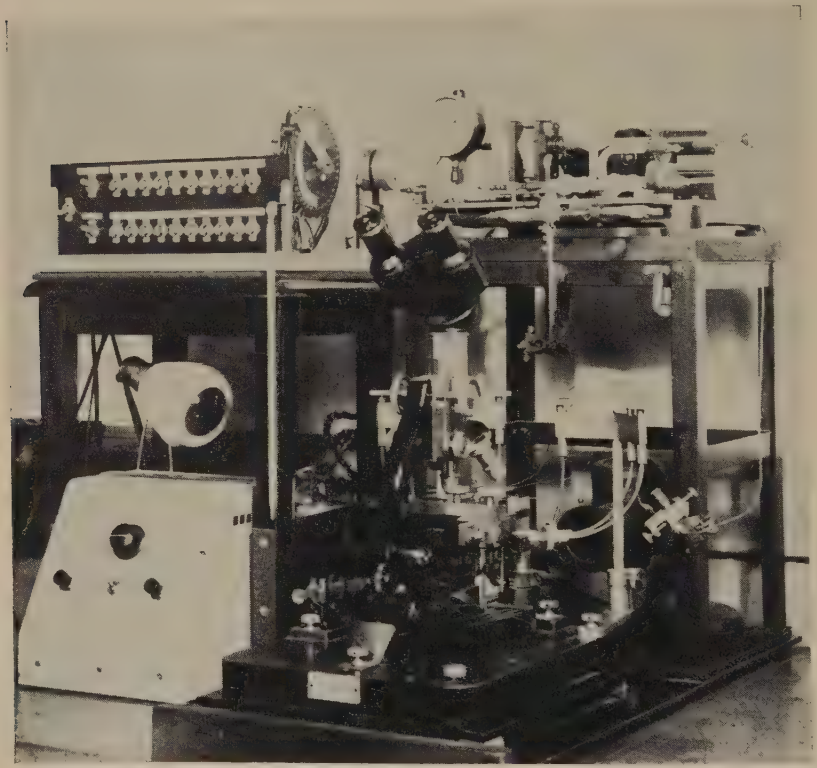

FIGURE 8. The actual apparatus is shown in the above photograph. The positions of the microscope, micromanipulator, and the drop-retractor were arranged to afford maximum convenience for operation.

To maintain time schedules, an electric timer was designed and built (mounted to the left of the micromanipulator and drop-retractor). This unit sets into operation the semi-automatic component (motor-driven syringe, FIGURE 7) of the drop-retractor at pre-determined time schedules, for example, at 2, 5, and 10 minutes after the interface is formed.

It will be noted that the timer as well as the drop-retractor are placed at eye level in order to facilitate observation of these instruments as well as to measure the oil drop visible in the microscopic field.

The optical equipment employed in measuring the oil drops includes: Leitz No. 5 objective, Zeiss 10× oculars, with micrometer disk, and a Zeiss 1.5× Bitukni attachment. These optical units provide a magnification of 450×.

diameters. These data indicate that proteins are still present in the cytolytic residue, but the amounts seem to decrease with time so that fewer molecules are available to the introduced interface. In fact, proteins may be detected by the drop-retraction method in the surrounding sea water when cytolyzed eggs have been left standing in a hanging drop for several minutes. It is obvious that the proteins have diffused out of the cytolyzed residue into the surrounding medium.

Such outward diffusion tends to diminish the concentration near the zone of the injected oil. It is reasonable to conclude that the maximum available concentration of protein occurs at the instant of cytolysis. At this time, protein adsorption on suitable oil-water interfaces may be readily demonstrated—by the formation of spontaneous Devaux effects.

Another factor that must be taken into account is the possible role of surface-denaturating agents (see FIGURE 15). Accordingly, the surface denaturation of cytoplasmic proteins would diminish with time providing the surface-denaturating agent (x in FIGURE 15) either diffused away or became bound by other products of cytolysis. This topic will be discussed in greater detail later on.

Another interesting application of the drop-retraction technique is shown by FIGURE 9,C and D. It is possible to microinject a drop of oil into the cytoplasm of a living *Asterias* oocyte and to maintain continuity between the oil drop and pipette. It will be noted in FIGURE 9,C that the oil drop was placed near the germinal vesicle without inducing the characteristic cytolytic reaction that usually follows any mechanical injury to this structure. After 10 minutes' exposure of the oil-water interface to the cytoplasm, the drop was retracted until a crinkling effect was obtained (FIGURE 9,B). The s/A value for this experiment was *ca.* 0.1. It will be further noted that the germinal vesicle remained intact and that a small cytolytic zone was present around the retracted oil drop.

Some surface denaturation of proteins occurred at this oil-water interface, even though the cell remained reasonably intact. The small cytolytic zone that surrounded the retracted oil drop probably accounts for all the protein that has become surface denatured. It is obvious that enough protein was not surface denatured to produce a spontaneous Devaux effect.

The changes that occur on cytolysis in the protein structure of the cell will be discussed later (see also FIGURE 15).

Measurements on Model Protein Systems

The interpretation of the properties of cytoplasmic proteins on the basis of spontaneous Devaux effects must depend on information obtained from a study of individual protein systems. Up to now, over 60 different organic phases have been investigated. The interfacial denaturation at oil-water interfaces of various proteins and nucleoproteins has also been measured.[5, 14, 15, 17] Included in the list of proteins are: bovine and human plasma albumins, thrombin (beta-globulin), crystalline trypsin and chymotrypsin, crystalline ribonuclease, hyaluronidase, protamine, protamine-nucleate complexes, liver nucleoproteins, thymus nucleohistone, tobacco mosaic virus nucleoprotein, and lyophilized cytoplasmic residue from sea-urchin eggs (fraction rich in cytoplasmic ribonucleoproteins).

The action of various chemical agents on interfacial denaturation of these proteins has also been investigated.* Of particular interest are the compounds that either enhance or inhibit surface denaturation. A few examples will be described, since these results may aid in the interpretation of the surface chemical behavior of proteins, in general, and of cytoplasmic

* Some of this material was also presented at the AAAS-Gibson Island Research Conference on Cancer, August 12, 1946, an abstract of which appeared in Cancer Research. 7: 44. 1947.

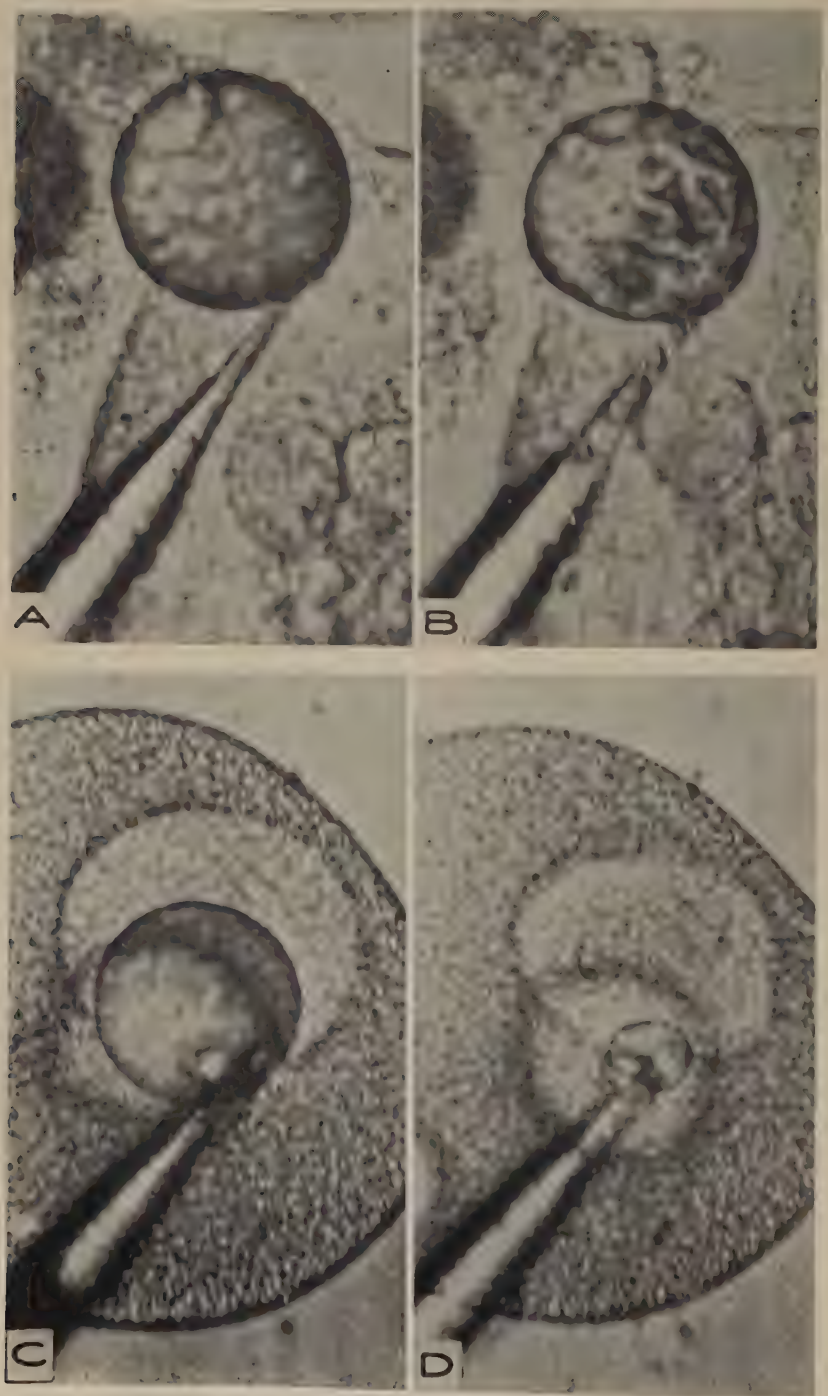

FIGURE 9. *(For description see facing page).*

proteins, in particular. Certain aspects of protein structure will also be discussed.

A. The Effects of Surface Forces and of Diamidines on the Surface Denaturation of Liver Nucleoproteins.

The following data are presented to illustrate the effects of different types of surface forces. The magnitude of the surface forces can be readily controlled by altering the nature of the oil-water interface, *e. g.*: different oil phase, or by changing the pH or ionic strength of the aqueous phase. In the following series, the surface forces were varied by employing different organic liquids to produce the oil-water interfaces.

These data also show the effects produced by a combination of factors, *e. g.*, surface forces + chemical agents. The aromatic diamidines, either stilbamidine* or propamidine†, at 0.001M, were added to the liver nucleoprotein. The concentration of liver nucleoprotein employed in the measurements summarized in FIGURE 10 was 1.25 mg./ml. The nucleoprotein was dissolved in a saline solution of ionic strength = 0.02, buffered at pH 7.7.

Tricaprylin + Hexadecanoic Acid‡-Water Interface. For liver nucleoprotein alone, the value of $k = 0.062$ and the slope, $a = 0.54$. In the presence of stilbamidine, k increased to 1.0. On the other hand, propamidine reduced the value of k to 0.0038. Indeed, the values of s/A were not measurable during the first 3 or 4 minutes. The lowest value of s/A that can be measured with the present experimental equipment is about 0.02.

Iso-amylaniline-Water Interface. The qualitative effects of the two diamidines on the surface denaturation of liver nucleoprotein resembled those obtained with tricaprylin + hexadecanoic acid. There were significant quantitative differences, however. There was more surface denaturation of liver nucleoprotein at this interface as shown by a k value of 0.16 ($a = 0.33$). Furthermore, the enhancing effect of stilbamidine or the depressing effect of propamidine on the surface denaturation of liver nucleoprotein was much less striking.

Ethyl Formyl Ethyl Xanthate-Water Interface. Considerable amounts of liver nucleoprotein were surface denatured at this interface, $k = 0.8$ and $a = 0.083$. Stilbamidine produced a slight increase while propamidine mildly depressed the surface denaturation. The surface forces at this interface are too high for liver nucleoprotein, consequently not much can be learned from the simultaneous action of surface forces and diamidine with this interfacial system.

* 4,4′-diamidinostilbene diisethionate.
† 4,4′-diamidinodiphenoxypropane diisethionate.
‡ The mol fraction of hexadecanoic acid in tricaprylin was 0.01.

FIGURE 9 (*See opposite page*). Application of drop-retraction technique to cells.
A. A micropipette with attached oil drop was placed into the cytolyzed residue near the margin of an *Asterias* oocyte, approximately 5 minutes after cytolysis. Note outpouring of granules through the torn membrane of oocyte.
B. After 10 minutes' exposure to the residue, the drop was retracted until an incipient crinkling was developed at the oil—water interface. In this experiment, the value of s/A, as calculated from the two surface areas (see FIGURE 7), is ca. 0.9.
C. An oil drop was carefully injected into the cytoplasm of an intact *Asterias* oocyte, near the region of the germinal vesicle (note the prominent nucleolus). The micropipette was not removed, so that continuity between the oil drop and the pipette could be maintained.
D. After 10 minutes' exposure, the oil drop was retracted until the crinkling stage was developed. The value of s/A in this experiment is ca. 0.1. Note the small cytolytic zone around a portion of the retracted oil drop produced by the original injection of the oil drop shown in C. The residue resulting from the small cytolytic zone was probably the source of the proteins surface denatured at this oil—water interface.

Ethyl Oleate-Water Interface. Although the surface denaturation of liver nucleoprotein at this interface was nearly identical to that observed with *iso-amylaniline-water* interfaces, the action of the diamidines was different. Both stilbamidine and propamidine depressed the surface denaturation of liver nucleoprotein at this interface. The depressing action of propamidine, however, was considerably greater than that produced by stilbamidine.

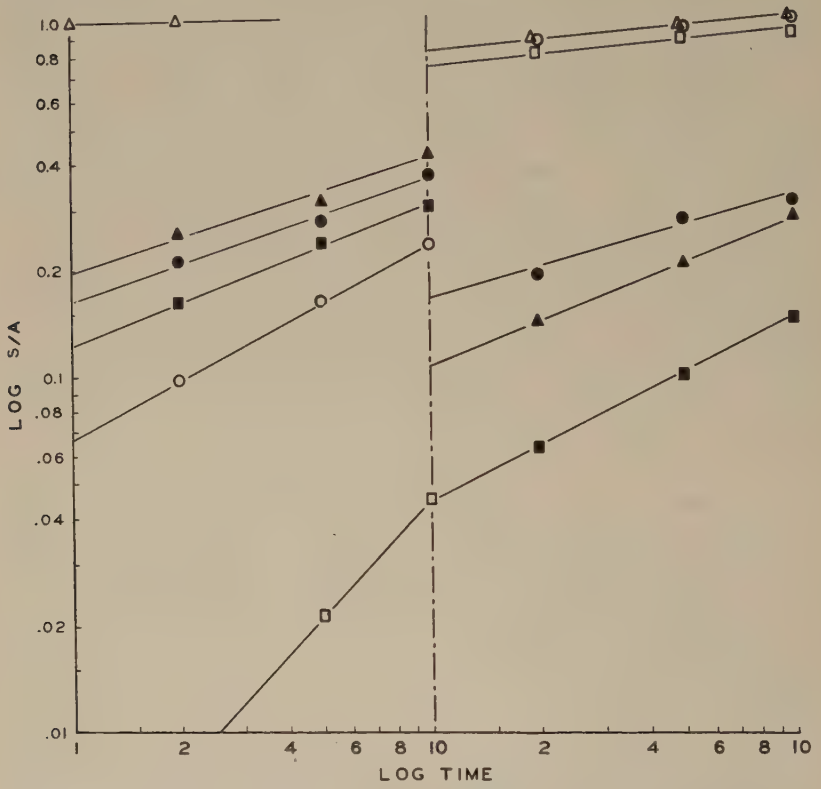

FIGURE 10. The effects of surface forces and aromatic diamidines on the surface denaturation of liver nucleoprotein. *Log s/A* vs. *log time*, in minutes.

Left graph. Tricaprylin (hexadecanoic acid)-water interface. *Open ovals:* LN*. *Open triangles:* LN + stilbamidine (0.001M). *Open squares:* LN + propamidine (0.001M). *Iso-amylaniline-water* interface. *Solid ovals:* LN. *Solid triangles:* LN + stilbamidine (0.001M). *Solid squares:* LN + propamidine (0.001M).

Right graph. Ethyl formyl ethyl xanthate-water interface. *Open ovals:* LN. *Open triangles:* LN + stilbamidine (0.001M). *Open squares:* LN + propamidine (0.001M). *Ethyl oleate—water* interface. *Solid ovals:* LN. *Solid triangles:* LN + stilbamidine (0.001M). *Solid squares:* LN + propamidine (0.001M).

* Liver nucleoprotein (LN) in all preparations: 1.25 mg./ml in NaCl-solution of ionic strength = 0.02, pH = 7.7.

These data indicate that surface forces as well as chemical agents are of importance in the surface denaturation of liver nucleoprotein. The most striking differences between the action of stilbamidine and of propamidine were produced at the *tricaprylin (hexadecanoic acid)-water* interface.

B. Action of Aromatic Diamidines and Other Compounds in Modifying the Stilbamidine Effect on Liver Nucleoprotein at the Tricaprylin (Hexadeconoic Acid)-Water Interface.

The data, summarized in FIGURE 11, were obtained with liver nucleoprotein (1.25 mg/ml.) dissolved in NaCl-solution of ionic strength = 0.02, buffered at pH 7.7 (Kopac[14]).

With stilbamidine, at concentrations as low as 0.0005M, the surface denaturation of liver nucleoprotein at the *tricaprylin* (*hexadecanoic acid*)-*water* interface increased from $k = 0.092$ to 0.215 and from $a = 0.34$ to 0.90. The following data show the action of other compounds on this

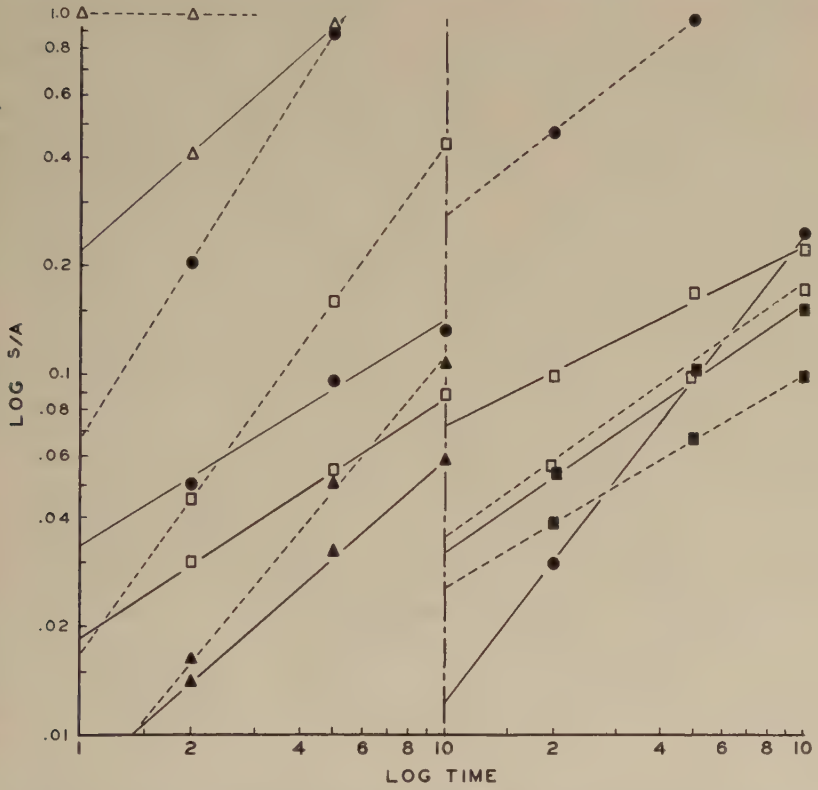

FIGURE 11. Action of aromatic diamidines and other compounds in modifying the stilbamidine effect on liver nucleoprotein at *Tricaprylin* (*hexadecanoic acid*)—*water* interface. *Log s/A* vs. *log time*, in minutes.
Left graph. Open triangles: LN* + stilbamidine (0.0005M). *Open squares:* LN + propamidine (0.0005M). *Solid ovals:* LN + phenamidine (0.0005M). *Solid triangles:* LN + pentamidine (0.0005M).
Right graph. Solid ovals: LN + colchicine (0.001M). *Open squares:* LN + 1,2-diphenylethylamine (0.001M). *Solid squares:* LN + 1,2-di-p-anisylethylamine (0.001M).
Broken lines, ---, indicate changes in *log s/A* following the addition of stilbamidine, at 0.0005M to each preparation.
* Liver nucleoprotein (LN) in all preparations: same as in FIGURE 10.

effect. The measurements were obtained by adding stilbamidine, at 0.0005M, to liver nucleoprotein previously treated with one of the other compounds.

Aromatic Diamidines. The aromatic diamidines were employed at concentrations of 0.0005M. On adding 0.0005M of stilbamidine to a preparation already containing this amount (total concentration = 0.001M), the surface denaturation increased to $k = 1.0$.

Phenamidine*, propamidine, and pentamidine†, depressed the stilbamidine effect in proportion to their individual depressing actions on the surface denaturation of liver nucleoprotein. The k values were the following: pentamidine = 0.0068, propamidine = 0.165, and phenamidine = 0.64.

With propamidine or pentamidine, the k values were lower with stilbamidine added, but the slopes were much higher. The slope, a, for propamidine increased from 0.67 to 1.41 and for pentamidine from 0.88 to 1.48. The k value for phenamidine was increased on adding stilbamidine, from 0.31 to 0.64, and the slope, a, was likewise increased, from 0.62 to 1.63.

In the data summarized in FIGURE 11, stilbamidine was added after the other diamidines were allowed to act on the liver nucleoprotein. The question came up whether or not any of these compounds could block the stilbamidine effect if they were added after the nucleoprotein had been exposed to stilbamidine. One such series was measured with propamidine and stilbamidine. In one set, propamidine was added following stilbamidine, and in another, stilbamidine was added following propamidine. The k values for the two systems were nearly identical. The slope, a, of the curve for stilbamidine + propamidine was slightly lower than for the curve of propamidine + stilbamidine (1.26 and 1.44, respectively).

Colchicine. Colchicine, at 0.001M, + stilbamidine promoted a higher degree of surface denaturation than stilbamidine alone. Stilbamidine increased the k-value of colchicine from 0.12 to 0.29 (compared with 0.21 for stilbamidine alone), but the slope, a, was reduced from 1.33 to 0.93. Colchicine was the only compound of the many tested that augmented the surface denaturating action of stilbamidine on liver nucleoprotein.

It will be noted (FIGURE 11) that colchicine alone, at 0.001M, produced a k-value of 0.013 and a slope, a, of 1.33. Thus, protein unfolding was inhibited at first, but this effect was rapidly dissipated as shown by the high value of the slope, a. In general, the value of k represents initial adsorption and preliminary unfolding of the proteins that are exposed to interfacial forces for one minute. The value of the slope, a, is generally assumed to be a measure of the rate of unfolding of the initially adsorbed protein molecules at the oil-water interfaces (Kopac[5]).

The high slopes produced by colchicine, however, could be realized only if additional molecules unfolded at the interface, since the low value of k would not account for the increased interfacial area, s, occupied by the surface denatured proteins, for example, at time = 10 minutes. This represents a 19-fold increase in the area, s, and k could account for only about 20 per cent of this increase.[5]

The curves of depressor diamidines + stilbamidine have slopes similar to those measured with colchicine. These data suggest that some linkages can be weakened, *i.e.*, by stilbamidine, while others can be strengthened, *i.e.*, by propamidine, pentamidine, or by phenamidine (see also discussion of tobacco mosaic virus nucleoprotein). When two diamidines of different properties are present simultaneously, the combined effect will be a resultant

* 4,4′-diamidinodiphenylether dihydrochloride.
† 4,4′-diamidinodiphenoxypentane dihydrochloride.

between the weakening characteristics of stilbamidine and the strengthening characteristics of the other diamidines.

Thus, the combined action of propamidine + stilbamidine, for example, quantitatively duplicates the effects produced by colchicine. Colchicine, therefore, appears to have double action, *i.e.*, simultaneous weakening and strengthening action of side chain linkages, with the former effect predominating when stilbamidine is present.

On the other hand, colchicine may inhibit initial adsorption (responsible for low k-values) and unfolding of liver nucleoprotein molecules, but it does not prevent accumulative adsorption and subsequent unfolding (responsible for high a-values). Possibly, colchicine has an interfacial effect that might prevent extensive adsorption, while at the same time it weakens the protein molecules so that they readily unfold when they reach the interface and are exposed to interfacial forces.

Phenethylamines. These compounds form a class by themselves. Either 1,2-diphenylethylamine or 1,2-di-p-anisylethylamine, at 0.001M, protected the liver nucleoprotein molecules against stilbamidine to such an extent that the surface denaturation of liver nucleoprotein was even lower in the presence of stilbamidine than in its absence. Both compounds yielded significantly lower k values on the addition of stilbamidine. 1,2-diphenylethylamine produced a higher slope on addition of stilbamidine, increasing it from 0.51 to 0.74. 1,2-di-p-anisylethylamine, however, yielded a lower slope, decreasing it from 0.70 to 0.60.

1,2-diphenylethylamine, at 0.001M, did not show any appreciable action on the surface denaturation of liver nucleoprotein. On the other hand, 1,2-di-p-anisylethylamine, at the same concentration, depressed interfacial denaturation to the same degree as phenamidine, at 0.0005M (FIGURE 11).

In the absence of other tests, it could be concluded that either 1,2-di-p-anisylethylamine or phenamidine have a similar action on the liver nucleoprotein molecules. However, on the addition of stilbamidine, at 0.0005M, to the preparations containing either compound, the differences in surface denaturation were striking. Phenamidine + stilbamidine produced a higher surface denaturation than phenamidine alone. On the other hand, 1,2-di-p-anisylethylamine + stilbamidine depressed surface denaturation to a level lower than that caused by 1,2-di-p-anisylethylamine alone. Obviously, phenamidine and 1,2-di-p-anisylethylamine do not have the same action on the nucleoprotein molecules.

The action of these two phenylethylamines on liver nucleoprotein distinguishes them from all other compounds tested. The situation is unusual, in that the addition of stilbamidine actually increases the depressor action of another compound.

More data, particularly on the action of the phenylethylamines on albumin and other proteins will be needed before the curious combined actions of 1,2-diphenylethylamine + stilbamidine or of 1,2-di-p-anisylethylamine + stilbamidine can be explained. The nucleate fraction of the nucleoprotein may be partly responsible. Some suggestions about this effect will be discussed later (section on bovine plasma albumin). Furthermore,

several new aromatic amidines, in combination with nucleic acid, produced new and unpredictable effects on the surface denaturation of bovine plasma albumin (Kopac[15]).

C. The Action of Aromatic Diamidines and Other Compounds on the Surface Denaturation of Cytoplasmic Proteins.

The material employed in this series consisted of the proteins of the cytoplasmic matrix, mitochondria, and the smaller yolk granules that may be obtained by rapid disintegration of unfertilized sea-urchin (*Arbacia*) eggs in Ca-free media and by subsequent centrifugal fractionation of such products (Kopac[10]). The mitochondrial content was 5 to 10 times higher than that normally present in unfertilized *Arbacia* eggs. The cytoplasmic

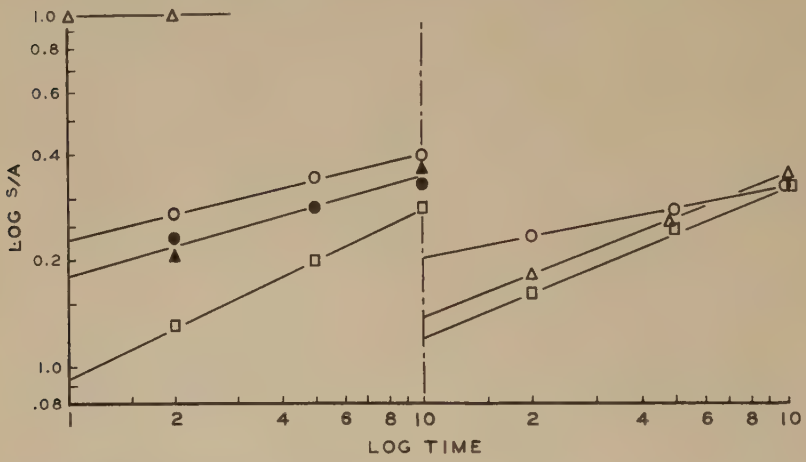

FIGURE 12. The action of aromatic diamidines on the surface denaturation of cytoplasmic proteins. *Log s/A* vs. *log time*, in minutes.

Left graph. Tricaprylin (hexadecanoic acid)-water interface.

Open ovals: CP*. *Open triangles:* CP + stilbamidine (0.001M). *Open squares:* CP + propamidine (0.001M). *Solid ovals:* CP + phenamidine (0.001M). *Solid triangles:* CP + bis-amidinomethyldibenzyl (0.001M).

Right graph. Tricaprylin—water interface.

Open ovals: CP. *Open triangles:* CP + stilbamidine (0.001M). *Open squares:* CP + propamidine (0.001M).

* Cytoplasmic proteins (CP): 2 mg./ml., lyophilized weight, in NaCl-solution of ionic strength = 0.15, pH = 7.5.

matrix residue consisted to a large extent of nucleoprotein complexes (see Harvey and Lavin[8]). Following centrifugal fractionation, the material was lyophilized.

The lyophilized cytoplasmic residue, at 2 mg./ml., was reconstituted by dissolving and suspending in a buffered, saline solution of ionic strength = 0.15. The saline solution was maintained at pH 7.5. The data are summarized in FIGURE 12.

Stilbamidine enhanced surface denaturation at the *tricaprylin (hexadecanoic acid)-water* interface, while the other diamidines depressed surface denaturation. Propamidine depressed considerably more than either phenamidine or bis-amidinomethyldibenzyl*. It is of interest to note that both

* 4,4′-bis-amidinomethyldibenzyl dihydrochloride.

phenamidine and bis-amidinomethyldibenzyl produced similar effects on tobacco mosaic virus nucleoprotein as well as on cytoplasmic proteins.

At the *tricaprylin-water* interface, the surface denaturating action of stilbamidine was considerably reduced (see also tobacco mosaic virus nucleoprotein). Both stilbamidine and propamidine temporarily depressed surface denaturation. Although their k-values (0.14 and 0.125, respectively) were lower than that obtained for cytoplasmic residue alone ($k = 0.20$), the slopes, a, were 0.38 and 0.40, respectively, compared to 0.20 for the cytoplasmic residue alone.

There is considerable evidence to indicate that some of the cytoplasmic proteins, including the mitochrondria, are high molecular weight complexes (Claude[19]) and that dissociation of these into smaller units is a prerequisite to subsequent surface denaturation. In several ways, the action of the diamidines on this material was similar to their action on tobacco mosaic virus nucleoprotein (see following section). Cytoplasmic proteins are highly complex and, at present, the simplest models appear to be the virus nucleoproteins.

D. The Action of Aromatic Diamidines on the Surface Denaturation of Tobacco Mosaic Virus Nucleoprotein (TMV) at Oil-Water Interfaces.

These data, summarized in FIGURE 13, show the action of several diamidines on a complex nucleoprotein in which the the molecular complex must be dissociated into smaller molecules before surface denaturation can occur (see Seastone[16]). The surface denaturation of complexes of this category, therefore, involves: (1) dissociation of the large molecules or complexes into smaller molecules and (2) unfolding of the smaller molecules by the action of surface forces.

Surface forces of appropriate magnitude may be sufficiently strong to induce both changes and if an agent enhanced the dissociation of the large complex, surface denaturation should be correspondingly increased. If such agents also facilitate unfolding, surface denaturation would be enhanced to an even higher degree.[14, 17]

The results differ from those previously described for liver nucleoprotein. The surface denaturation of TMV (1 mg./ml. in NaCl-solution, ionic strength = 0.02, pH = 7.7) was strikingly enhanced by either stilbamidine, propamidine, or pentamidine, each at 0.001M. The k values ranged from 0.96 to 1.0.

Phenamidine (0.001M) or bis-amidinomethyldibenzyl (0.001M), on the other hand, depressed the surface denaturation of TMV at the *tricaprylin (hexadecanoic acid)-water* interface (k-values, 0.034 and 0.017, respectively).

The action of surface forces on the denaturation of TMV has also been demonstrated. The surface denaturation of TMV + stilbamidine was tested with several oil-water interfaces. The k values were highest at the *tricaprylin (hexadecanoic acid)-water* interface, and much lower at *tricaprylin-water* or at *tricaprylin (stilbestrol)-water* interfaces (0.21, 0.014, and 0.011, respectively). These data indicate that stilbamidine does not enhance unfolding of the dissociation products as much at some interfaces as it does at others. A similar interfacial effect was observed with liver nucleoprotein + stilbamidine at the *ethyl oleate-water* interface (FIGURE 10).

Although propamidine or pentamidine generally depressed the surface denaturation of proteins at oil-water interfaces, their action on TMV may be interpreted in the following way: For example, propamidine, at 0.001M, enhanced the surface denaturation of bovine plasma albumin (BPA) providing the concentration of the latter was not higher than 2 mg./ml.[15] If the concentration of BPA was increased to 5 mg./ml., then propamidine

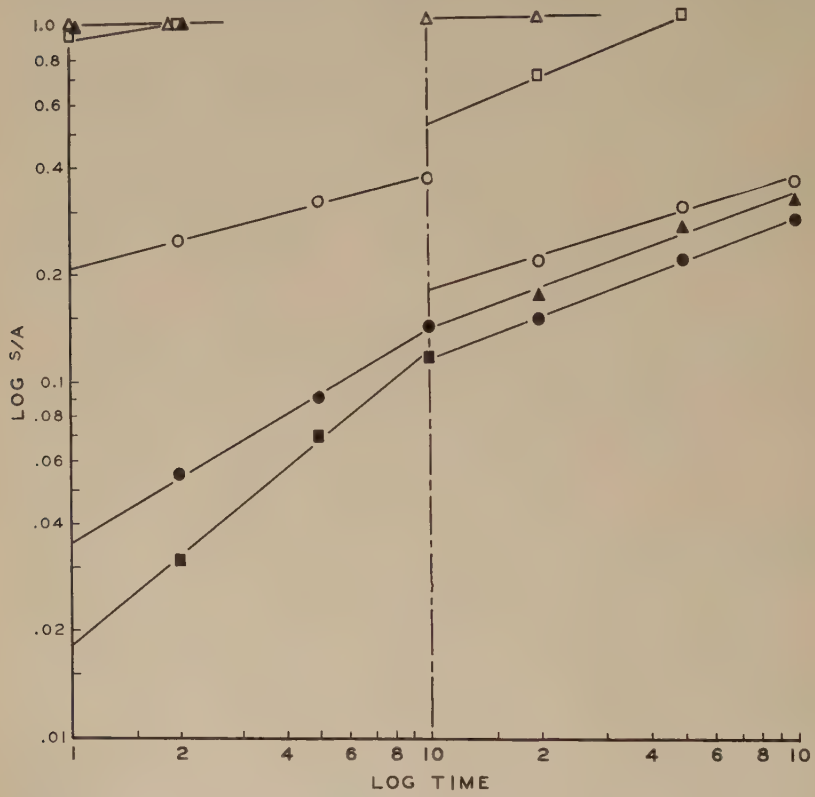

FIGURE 13. The action of aromatic diamidines on the surface denaturation of tobacco mosaic virus nucleoprotein at oil-water interfaces. *Log s/A* vs. *log time*, in minutes.

Left graph. Tricaprylin (hexadecanoic acid)-water interfaces. *Open ovals:* TMV* (1.0 mg./ml.). *Open triangles:* TMV + stilbamidine (0.001M and 0.0005M). *Open squares:* TMV + propamidine (0.001M). *Solid ovals:* TMV + phenamidine (0.001M). *Solid triangles:* TMV + pentamidine (0.001M). *Solid squares:* TMV + bis-amidinomethyldibenzyl (0.001M).

Right graph. Tricaprylin (hexadecanoic acid)-water interface. *Open ovals:* TMV (1.96 mg./ml.). *Open triangles:* TMV + stilbamidine (0.001M). *Open squares:* TMV + propamidine (0.001M). *Tricaprylin-water* interface. *Solid triangles:* TMV + stilbamidine (0.001M). *Tricaprylin (stilbestrol)-water* interface. *Solid squares:* TMV + stilbamidine (0.001M).

* Tobacco mosaic virus nucleoprotein (TMV): in NaCl-solution of ionic strength = 0.02, pH = 7.7.

depressed surface denaturation at *tricaprylin (hexadecanoic acid)-water* interfaces.

All three aromatic diamidines in this group may be expected to dissociate an appreciable fraction of the TMV complex (P^n) to smaller molecules (np),the latter being more susceptible to surface denaturating forces. If the concentration of np molecules is low, then propamidine or pentamidine

may enhance surface denaturation as observed with low concentrations of BPA.

It may, at first, be difficult to understand why one diamidine always enhances surface denaturation while others do so only if the *diamidine/np* (= *D/np*) ratio is high. It has been suggested (Kopac[17]) that stilbamidine enhances surface denaturation because it can weaken or destroy certain critical side chain linkages in the protein molecule.

Crammer and Neuberger[18] postulated that in the native ovalbumin molecule the phenolic groups are bound in linkage, possibly a hydrogen bond. It was suggested that the phenolic group may interact with a carboxyl group, via H-bridges, similar to interaction of phenols with salicylic acid. An amidine group may be expected to rupture a glutamyl-tyrosyl side chain linkage by interacting with the H-bridges. If this should happen, the trans-stilbene structure of stilbamidine could present only one amidine group/stilbamidine molecule for interaction with either the carboxyl or the phenolic groups of the severed side chain linkage. Indeed, if the concentration of stilbamidine were high enough, both end groups of the severed linkage could interact with a pair of amidine groups, but these would have to come from two stilbamidine molecules, and not one. It must be remembered that only one amidine group/molecule can be involved in a severed linkage, since the steric architecture of trans-stilbamidine does not readily permit other possibilities.

Propamidine or pentamidine, on the other hand, could interfere with the same type of side chain linkages, but now it is sterically possible for both amidine groups of one molecule to combine with the phenolic and carboxyl end groups of the severed side chain linkage. Under such conditions, the side chain linkage would not be weakened, rather the phenolic and carboxyl groups would be joined together by the propamidine or pentamidine molecules. Thus, the structure of the protein molecule is not weakened, and it is even possible that a glutamyl-propamidine-tyrosyl linkage might be stronger than the original glutamyl-tyrosyl linkage.

This situation might be expected if the *D/np* ratio were low. With a high *D/np* ratio, enough diamidine molecules are available so that the severed free groups of a glutamyl-tyrosyl linkage, for example, could be joined to two diamidine molecules instead of one. There would be no joining together of the carboxyl and phenolic groups to give a glutamyl-propamidine-tyrosyl linkage, but rather glutamyl-propamidine and tyrosyl-propamidine linkages could be formed. Since no stable junction between the free ends of the two diamidine molecules is to be expected, such a condition would weaken the protein molecule in the same way as proposed for stilbamidine.

That the *diamidine/TMV* ratio is important can be shown by the following data: Where the propamidine concentration was 0.001M and the TMV concentration 1 mg./ml., the value of k was 0.96. On doubling the concentration of TMV, for the same concentration of propamidine, the value of k fell to 0.50. On the other hand, the surface denaturating action of stilbamidine, at 0.005M, was as high on a TMV concentration of 2 mg./ml., as it was at 0.001M for one-half the concentration of TMV (k = 1.0, for both).

According to the general scheme outlined above, there is little, if any, possibility that stilbamidine can reinforce any linkage that it might break. For example, it is sterically impossible to produce a glutamyl-stilbamidine-tyrosyl linkage in the way that a glutamyl-propamidine-tyrosyl linkage can be formed. At all concentrations, stilbamidine weakens the architecture of globular proteins.

Both phenamidine or bis-amidinomethyldibenzyl structures could form linkages as described for propamidine and pentamidine. However, judging from the action of these molecules on the surface denaturation of TMV, it would appear that the TMV complexes are strengthened so that surface forces are unable to dissociate appreciable fractions of the giant molecules. Accordingly, surface denaturation of TMV is depressed by these two compounds.

Also, owing to the structure of these two molecules, it would appear that they could readily form glutamyl-phenamidine-tyrosyl or glutamyl-amidino-methyldibenzyl-tyrosyl linkages, and that it would be difficult for two diamidine molecules to react with the two liberated terminal groups of the side chains even if the diamidine concentration were high. This seems to be especially true for bis-amidinomethyldibenzyl.[5] Thus, these two substances not only protect the large TMV complexes from dissociating, but they also protect the dissociation products from surface denaturating forces. Either condition would account for the depressing action of these compounds on the surface denaturation of TMV.

Bis-amidinomethyldibenzyl, at 0.001M, completely inhibited the surface denaturation of BPA, at 2 mg./ml., and of crystalline ribonuclease, at 1 mg./ml. With higher concentrations of BPA (5 mg./ml.), bis-amidinomethyldibenzyl strongly depressed surface denaturation, but it did not prevent it, as was observed with lower concentrations of BPA. Owing to the structure of this compound, it would appear that such molecules always react with two side chains to form a new stable side chain linkage *e.g.*, glytamyl-amidinomethyldibenzyl-tyrosyl linkage. Accordingly, BPA molecules with glutamyl-amidinomethyldibenzyl-tyrosyl linkages are more resistant to surface denaturation than BPA molecules with glutamyl-tyrosyl linkages.

If 0.0005M of stilbamidine was added to TMV treated with either phenamidine or bis-amidinomethyldibenzyl, the resulting surface denaturation was the same as with stilbamidine alone ($k = 1.0$). This is similar to the combined action of stilbamidine and bis-amidinomethyldibenzyl on BPA in which the stilbamidine effect predominated. Indeed, bis-amidinomethyldibenzyl produced no measurable inhibition of the stilbamidine effect on BPA. Obviously, stilbamidine can replace either phenamidine or bis-amidinomethyldibenzyl in the TMV complex, either before or after dissociation.

E. The Action of Aromatic Diamidines on the Surface Denaturation of Bovine Plasma Albumin (BPA) and the Modification of These Effects by Nucleic Acids.

The following data, summarized in FIGURE 14, were based on measurements of diamidine on the surface denaturation of bovine plasma albumin (5 mg./ml., dissolved in NaCl solution of ionic strength = 0.02, buffered at

pH 7.7). All measurements were made with the *tricaprylin* (*hexadecanoic acid*)-*water* interface. These measurements are of particular interest since they illustrate clearly that the action of stilbamidine in enhancing, and other substances in depressing, surface denaturation results from the simultaneous action of surface forces and of chemical agents on the protein molecules.

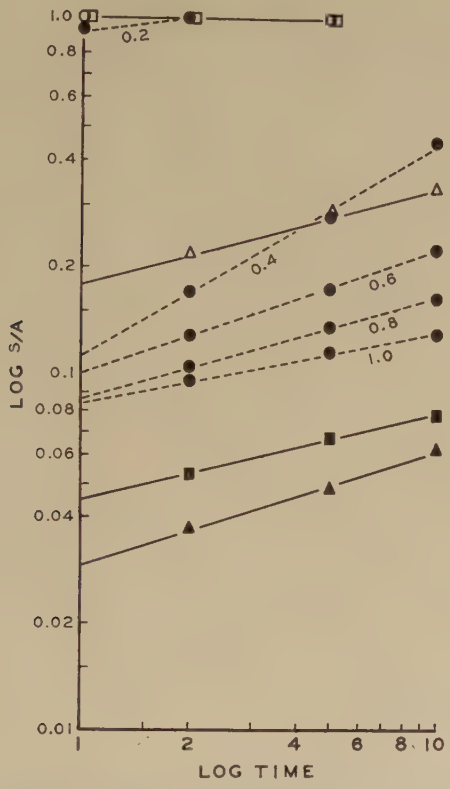

FIGURE 14. Action of stilbamidine, protamine, and nucleic acids on the surface denaturation of bovine plasma albumin at the *Tricaprylin* (*hexadecanoic acid*)-*water* interface. *Log s/A* vs. *log time*, in minutes. *Open ovals:* BPA* + stilbamidine (0.001M). *Solid ovals:* BPA + stilbamidine (0.001M) + Na ribonucleate (0.2 to 1.0 mg./ml.). *Solid triangles:* BPA + protamine (1 mg./ml.) + Na ribonucleate (1.0 mg./ml.). *Open squares:* BPA + protamine (1 mg./ml.) + stilbamidine (0.001M). *Solid squares:* BPA + protamine (1 mg./ml.) + stilbamidine (0.001M) + Na ribonucleate (1 mg./ml). *Open triangles:* BPA + protamine-ribonucleate (2 mg./ml.) + stilbamidine (0.001M).

* Bovine plasma albumin (BPA): 5 mg./ml, in NaCl-solution of ionic strength = 0.02, pH 7.7.

It will be noted that stilbamidine, at 0.001M, enhanced the surface denaturation of BPA as it did for liver nucleoprotein, TMV, crystalline ribonuclease, and the cytoplasmic proteins. On adding Na ribonucleate, the action of stilbamidine was neutralized, owing to the formation of a stilbamidine-ribonucleate complex. Na ribonucleate, at 0.2 mg./ml., had a very slight effect in reducing the surface denaturating action of stilbamidine; however, at 0.8 to 1.0 mg./ml., the action of stilbamidine was almost entirely neutralized[15]. The action of stilbamidine on BPA could be partly neutralized also by yeast adenylic acid (1 mg./ml.).

On the other hand, protamine, at 1.0 mg./ml., inhibited the surface denaturation of BPA. The surface denaturation of BPA, on the addition of Na ribonucleate, at 1.0 mg./ml., was considerably higher indicating that an appreciable fraction of the protamine was bound by the formation of a protamine-ribonucleate complex and thereby neutralized.

A typical stilbamidine effect was produced on adding stilbamidine, at 0.001M, to a preparation containing BPA (5 mg./ml.) + protamine (1 mg./ml.). On the subsequent addition of Na ribonucleate, at 1 mg./ml., a typical protamine effect was elicited indicating that stilbamidine was preferentially bound by the ribonucleate.

Stilbamidine, when added to preparations containing BPA (5 mg./ml.) + colloidally dispersed, protamine-ribonucleate (2 mg./ml.) produced an important effect. The action of stilbamidine was much lower than measured previously for stilbamidine + protamine. These data suggest that the protamine-ribonucleate complex was dissociated by stilbamidine to form a new stilbamidine-ribonucleate complex (Kopac[17, 20, 21]). Thus, the stilbamidine was removed from the reactants and protamine was released from the initial protamine-ribonucleate complex. Accordingly, the surface denaturation of BPA was much less than would be expected if stilbamidine, at 0.001M, alone were acting on the protein molecules.

Previous measurements have shown that protamine can also become trapped at the *tricaprylin (hexadecanoic acid)-water* interface providing it has been treated with stilbamidine.[17] It will be noted that the surface denaturation curve of BPA + protamine-ribonucleate + stilbamidine, in FIGURE 14, is approximately a sum of the curves for BPA + stilbamidine + ribonucleate (0.6 mg./ml.), for BPA + protamine + stilbamidine + ribonucleate, and for BPA + protamine + ribonucleate. It is apparent that the interface contained both surface denatured BPA and protamine.

A stilbamidine concentration of 0.0002M would be required to produce the same degree of surface denaturation with BPA alone. On this basis, about 80 per cent of the stilbamidine was removed by cleavage of the protamine-ribonucleate and subsequent formation of stilbamidine-ribonucleate[5].

These measurements are helpful in interpreting the spontaneous Devaux effect that occurs in *Asterias* oocytes after cytolysis. The first major point is that the surface denaturation of a protein can be significantly modified by the presence of certain chemical agents. On one hand, surface denaturation can be enhanced (stilbamidine), while on the other hand, the surface denaturation can be almost abolished (protamine). Stilbamidine has the action of a surface denaturating factor, while protamine behaves as an anti-surface denaturating factor.

Another major point is that the simultaneous presence of surface denaturating and anti-surface denaturating factors may produce an effect on surface denaturation that will depend on which of the two factors predominates. In the experiments reported here, one of the factors may be selectively removed by a third substance which by itself has no action on surface denaturation.

These data indicate that certain agents enhance interfacial denaturation because they weaken side chain linkages in protein molecules. No appreciable increase in interfacial denaturation was observed if these agents were removed before exposing the proteins to interfacial forces. The increased denaturation, therefore, results from the simultaneous action of surface forces with the chemical agents.[17]

If the proposed mechanism of action of stilbamidine is correct (see discussion on TMV), then the action of Na-ribonucleate is to remove stilbamidine from the protein molecules. On removal of the diamidine, the linkages which were previously broken by stilbamidine must have the capacity of reforming in such a way that the protein, even though once treated with stilbamidine, has approximately the same resistance to surface denaturing forces it possessed before stilbamidine was applied.

Presumably, not many side chain linkages are broken by stilbamidine, but these may be sufficiently important so that, on superimposing surface forces, the stilbamidine-treated molecule becomes considerably unfolded. In this connection, Crammer and Neuberger[18] pointed out that phenolic groups may be largely, but not exclusively responsible for the configuration of native ovalbumin molecules. On the basis of the toxicity of BPA + stilbamidine, to kidney tubules in tissue culture, Kopac[17] has suggested that phenolic groups may be released by the action of stilbamidine on the BPA molecule.

Interpretation and Significance of the Spontaneous Devaux Effect

An explanation of the Devaux effect as demonstrated in the *Asterias* oocyte may also explain the way in which proteins are organized in living cells. The surface chemical properties of cytoplasmic proteins can be summarized in three statements: (1) little, if any, surface denaturation occurs at experimentally introduced oil-water interfaces in contact with cytoplasm; (2) maximum surface denaturation, at appropriate oil-water interfaces, can be demonstrated when the oils are administered at the instant of cytolysis; and (3) the surface denaturation of cytoplasmic proteins diminishes with length of the post-cytolytic period.

The following scheme, obviously over-simplified, was devised to account for the Devaux effect and other surface chemical properties of cytoplasmic proteins. The interpretations are based largely on the information obtained from a study of model systems. The modification in surface denaturation of albumin and other proteins by certain aromatic diamidines, for example, indicates the important role of low molecular weight substances in maintaining the stability of protein molecules. Furthermore, these diamidines react with nucleic acid and with nucleotides to form inactive complexes so that their action on surface denaturation may be completely neutralized.

In the scheme outlined in FIGURE 15, the cytoplasm, consisting of matrix as well as granules, cytolyzes by disintegrating into a variety of complex substances of which two are specifically indicated. One of these is a nucleoprotein complex, $N_a P^n_a xy$, that contains, *inter alia*, nucleic acid, N_a, a complex protein of the type exemplified by viruses, P^n_a, a surface denaturating

factor, x, and an anti-surface denaturating factor, y. The other substance is also a nucleoprotein, N_bP_b, but in this complex, the protein moiety is probably of low molecular weight, similar to the protamines or histones. Some of the N_bP_b complexes may even be the split products of the $N_aP_a^nxy$ complexes.

The aggregation of the complexes, $N_aP_a^nxy$ and N_bP_b, to form an ultrastructure of the cytoplasmic matrix may be of two types: (1) spherical aggregates, the protein complexes rich in phospholipids and ribonucleic acids, and (2) linear aggregates, the protein complexes essentially rich in ribonucleic acids. Of the two, the spherical aggregates appear to have a greater stability since these are the sub-microscopic particles that can be

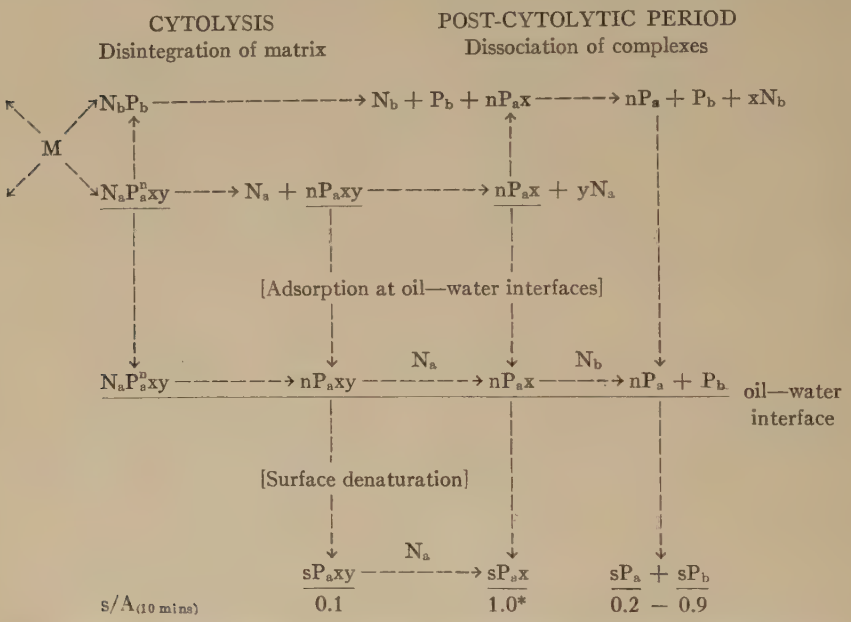

FIGURE 15. Schematic disintegration of cytoplasmic matrix into labile complexes and relation to Devaux effect.
The complexes, sP_axy, sP_ax, sP_a, and sP_b refer to surface denatured proteins. The other symbols are discussed in text.
* $s/A = 1.0$ in less than 1 minute, the spontaneous Devaux effect.

isolated, from various cells, under appropriate conditions, by centrifugal fractionation (Claude[19]; Brachet[23]). These aggregates or particles also possess a variety of enzymatic activities (Brachet[23]).

The linear aggregates may resemble the insulin fibrils or filaments described by Waugh,[24] except in cytoplasm such filaments may consist mainly of high molecular weight protein complexes of the virus type. Furthermore, these filaments may be much more labile than the insulin filaments, since this type of aggregate has not, as yet, been isolated from cytoplasmic residue.

At present, it is assumed that both types of complexes can be arranged in linear as well as spherical aggregation. On cytolysis, the linear aggregates

may be the first to disintegrate and to release the $N_aP^n_axy$ and N_bP_b complexes. According to Brachet, the low molecular weight fraction (supernate) of preparations from which the cytoplasmic sub-microscopic particles can be isolated, by centrifugal fractionation, contains a great deal of ribonucleic acid, much of which could be in the form of N_bP_b complexes. The spherical aggregates probably disintegrate during the early post-cytolytic periods, unless the cytoplasmic residue is treated to prevent such disintegration.[19, 23]

The nucleoprotein complex, N_bP_b, is assumed to be more stable than the $N_aP^n_axy$ complex, and its dissociation occurs after the onset of cytolysis, the probable half-life being of several minutes' duration. The other complex, on the other hand, may dissociate at the onset of cytolysis with a probable half-life period of seconds.

The complex, $N_aP^n_axy$, may dissocate progressively to form two fragments, nucleic acid and the protein components with adhering x and y substances. Along with this dissociation, the high molecular weight protein must dissociate into smaller molecules, nP_a. As the nucleic acid becomes released from the protein moiety, it can now attach to itself, the y or anti-surface denaturating factor.

There is no reason for doubting that the nucleoprotein complex, $N_aP^n_axy$, becomes adsorbed at appropriate oil-water interfaces. It is doubtful, however, whether this complex undergoes surface denaturation owing to the macromolecular state of the protein and to the presence of the anti-surface denaturing factor, y.

Similarly, the complex, P^n_axy, may be expected to become adsorbed at oil-water interfaces. Judging from the experimental data (see FIGURE 9,C and 9,D), no appreciable surface denaturation of this complex occurs. Although the low molecular weight proteins are more susceptible to surface denaturation, the presence of y suppresses this reaction.

With the appreciable release of nucleic acid, N_a, and subsequent binding of y to form an inactive yN_a complex, the remaining residue, nP_ax, is highly susceptible to surface denaturation. Here the proteins are of low molecular weight and in the presence of a surface denaturating factor, x. At this point, a spontaneous Devaux effect can be obtained at appropriate oil-water interfaces.

Thus, the spontaneous Devaux effect is made possible by the dissociation of complex nucleoproteins into nucleic acid and lower molecular weight protein molecules. Moreover, the rapid unfolding of adsorbed proteins at oil-water interfaces is enhanced by the removal of factors that normally maintain structural stability of the proteins by protecting them against surface and other denaturating forces.

Both high and low molecular weight proteins may be weakened or strengthened against surface denaturation by appropriate chemical agents. For example, stilbamidine enhances the surface denaturation of all proteins, so far tested. On the other hand, several agents (phenamidine, bis-amidinomethyldibenzyl, protamine, *etc.*) can protect macromolecular nucleoproteins (TMV), cytoplasmic proteins (isolated from sea-urchin eggs), and simple proteins against surface denaturation. It should be noted that 1,2-di-p-anisylethylamine + stilbamidine inhibits the surface denaturation

of liver nucleoprotein, more so than does either substance alone[14]. This example approaches the situation postulated in the *nPxy* complex.

The granular components of the *Asterias* oocyte, namely, mitochondria, yolk, and others, are not essential factors in the production of spontaneous Devaux effects.* For example, the most rapid development of the Devaux effect occurs on injecting an indicator oil drop into the optically homogeneous, germinal vesicle.

Significant amounts of surface denaturation can occur only if the nucleoprotein complexes dissociate with loss of the protecting *y*-factor. The protecting factor may either diffuse away, or else it may become bound to other agents released on cytolysis. On the basis of model experiments, the binding of the *y*-factor by nucleic acids seems the more probable.

The experimental data summarized, in FIGURE 14, suggest one possible mechanism. The surface denaturation of bovine albumin (5 mg./ml.) was almost completely inhibited by protamine (1 mg./ml.). On adding Na ribonucleate, much of the protamine was removed by formation of a colloidally dispersed, protamine-nucleate and, consequently, the albumin then became susceptible to surface denaturation. Thus, the removal of a protecting agent increased the susceptibility of the protein to surface denaturation.

The third statement suggests that cytoplasmic proteins have been reduced to smaller proteins, ordinarily the more stable molecules. The rapid development of spontaneous Devaux effects at the instant of cytolysis can best be explained by assuming the breakdown of unprotected macromolecular complexes, with a release of agents that enhance surface denaturation. Thus, along with the binding of y by nucleic acid, the activity of x now becomes possible. The action of x, a surface denaturating factor, would resemble the action of stilbamidine.

As the post-cytolytic periods are prolonged, the surface denaturation of the protein residue is less pronounced. This can be shown by the progressively lower s/A values, as measured by drop-retraction methods (see FIGURE 9,A and B). Such an effect might be expected if the x substances either diffused away or became bound by other products of cytolysis. For example, if stilbamidine is bound by nucleic acid, the proteins previously exposed to stilbamidine become less susceptible to surface denaturation.

The dissociation of the simpler N_bP_b complexes becomes of significance during the post-cytolytic period. The breakdown of this complex releases additional nucleic acid, N_b, which can then bind the surface denaturating factor, x, through the formation of the xN_b complex. The protein moiety, P_b, released on dissociation of the nucleoprotein may become adsorbed and surface denatured if of the histone, or more complex, type. If, on the other hand, the protein is of the protamine type, the adsorption and subsequent surface denaturation of the P_a type proteins may be inhibited. This would lead to lower s/A values as shown by the measurements of this quantity during late post-cytolytic periods.

* Under appropriate conditions, the disintegration of the granular components obtained from living cells may be prevented.[10,21]

The mechanism of inhibition by the protamine types of proteins is perhaps largely concerned with the interface rather than with the protein. Protamine undergoes some surface denaturation, but such unfolded molecules do not remain trapped at the interface except in special instances (Kopac[17]). Instead, they become displaced with increasing surface pressures, with the result that the measured values of s/A become exceedingly low (see action of protamine on surface denaturation of bovine albumin). Furthermore, such proteins may prevent the adsorption of those protein molecules that ordinarily would unfold and become trapped at the interface.

Thus, the protective action on proteins by anti-surface denaturating agents may be produced by two separate mechanisms. On the one hand, the agent may fortify strategic side chain linkages in the protein molecule, thereby protecting it against the action of surface forces. On the other hand, the agent may prevent the susceptible protein from reaching the interface. In either instance, the surface denaturation of the protein would be appreciably reduced.

Other Properties of Cytoplasmic Proteins

Other data also indicate that cytoplasmic proteins in intact cells are different from the isolated proteins. Pflüger[25] differentiated between the dead, or storage proteins, and the live, cytoplasmic proteins which he termed *lebendiges Eiweiss*.

Pollack[26] reported that solutions of picric acid when injected into amebas produced a coagulating action only if a local injury was produced at the site of injection. The same concentrations of picric acid, however, readily coagulated proteins, *in vitro*. These data might indicate that the proteins in intact amebas cannot be coagulated by picric acid, unless some cytolysis has been induced by the microinjection. Needham[27] has commented on the possible significance of Pollack's experiments, but he also cautioned that the neutralization of picric acid by cytoplasmic components might also be a factor. As mentioned earlier, the injection of trichloracetate into intact *Asterias* oocytes produced immediate fixation without the appearance of typical cytolytic reactions.

Perhaps the most suggestive data that cytoplasmic proteins of living cells differ from the isolated proteins have been presented by Vlés and Gex.[28] The ultra-violet spectrophotometric curves of intact sea-urchin eggs were shown by Vlés and Gex to differ strikingly from the absorption curves of eggs cytolyzed by hypotonic solutions or by crushing. The absorption curves of the cytolyzed eggs resembled the absorption curve of a solution of ovalbumin. These differences in absorption spectra between living and cytolyzed sea-urchin eggs suggest that cytoplasmic proteins are structurally different from those recovered in cytolyzed eggs.

It is well known that living cells are not attacked by pepsin or by trypsin, whereas dead cells are rapidly digested. Fermi[29] concluded that the configuration of the protein molecule in the living cells was different from that after death of the cell and that the "living molecule" could not be attacked by the enzyme.

Northrop[30] showed that neither pepsin nor trypsin penetrated living cells. The injection of large volumes of active trypsin solutions into amebas initiated rapid streaming and the injected solution collected into a spherical, granule-containing blister. Though the blister might be pinched off, the ameba became motionless and disintegrated within a few hours.

The microinjection experiments suggested but did not prove that living cells are susceptible to trypsin hydrolysis providing the enzyme reaches the cytoplasm (Northrop[30]). Northrop[31] later admitted that the microinjection data "were not entirely convincing owing to the difficulty of performing the operation without injury to the cells."

In view of the surface chemical behavior of cytoplasmic proteins, before and after cytolysis, the resistance of living cells to proteolytic enzymes is to be expected, since these proteins seem to be protected against certain denaturating forces as long as the cells remain intact. The resistance of native protein molecules to proteolytic enzymes is fairly well established (Neurath, Greenstein, Putnam, and Erickson[32]). Furthermore, the virus nucleoproteins are not attacked by proteolytic enzymes to any appreciable extent (Pirie[33]).

Accordingly, Fermi's concept that 'living molecules' are responsible for the resistance of living cells to proteolytic enzymes may be reinterpreted on the basis that cytoplasmic proteins in the intact cell cannot be readily denatured and that unless denaturation does occur, proteolytic activity will be significantly blocked.

It is obvious that the problems of cytoplasmic proteins cannot be entirely answered with available information. We do have certain clues, however, and micro-surface chemical methods offer one way in which additional information can be obtained.

The study of surface chemical behavior of simple protein preparations, at various oil-water interfaces, together with modifications of these properties by various chemical agents, is a step in this direction.

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SALIVARY GLAND CHROMOSOMES

By Ethel Glancy D'Angelo
Queens College, Flushing, New York

The cytogenetic implications of the structure of the salivary gland chromosomes of dipteran larvae, together with their large size and visibility *in vivo*, have prompted many investigators to use these chromosomes for studies on structure and physical properties. The characteristic appearance of the salivary chromosome, with its distinctive pattern of chromatic bands and achromatic interband regions, is now well known. It is in regard to the finer details of structure, however, that much controversy exists. At least five hypotheses are current regarding the structure of these giant chromosomes: (1) the alveolar concept proposed originally by Metz and Lawrence,[1] who believe that the striations seen in smear preparations are merely the result of drawing out the walls of alveoli and are not chromonemata, which to them are submicroscopic; (2) the polytene or multiple thread concept of Bauer[2] and of Painter and Griffin,[3] who maintain, respectively, that the visible striations are single chromonemata or bundles of chromonemata; (3) the view of Kodani,[4] based on alkali-urea treatment of the chromosomes, that there are only four chromonemata; (4) that of Ris and Crouse,[5] to the effect that the salivary chromosome consists of helically coiled chromonemata and that the alleged chromomeres are misinterpretations of these coils; and, finally, (5) that of Hinton,[6] that the bands are isolated groups of genic material separated from neighboring bands only by space, perhaps filled with nucleoplasm, and surrounded by a sheath.

There is generally more accord among investigators regarding the physical properties of these chromosomes. The findings of Vonwiller and Audova,[7] Barigozzi,[8] Stefanelli,[9] Pfeiffer,[10] and Buck[11] have indicated the salivary chromosomes to be tough, viscid, elastic gels. These observations are in essential agreement with those originally made by Chambers and Sands[12] on the chromosomes of *Tradescantia* and those by Chambers[13] on *Dissosteira* spermatocytes. These studies were extended by Buck,[11] who reported more detailed findings on the extensibility and elasticity of osmic vapor-treated salivary chromosomes. The experiments of Duryee[14, 15] on amphibian chromosomes indicated clearly how important it is that proper precaution be taken, inasmuch as the physical properties of the chromosomes could be modified extensively by the presence of either torn cytoplasm or calcium ions.

The present report will describe various observations made on the structure and physical properties of the salivary chromosome in the fresh state as determined by the technique of micromanipulation. The genus chosen for study was *Chironomus*, which is characterized by a flat salivary gland, one cell layer thick, in which the four chromosomes are plainly visible. The chromosomes are of especially large size, the largest being about 20 μ in diameter and approximately 150 μ in length. These factors are obviously important in facilitating micromanipulation.

It must be admitted freely at the outset that no criterion exists which can be used to decide indisputably whether the salivary chromosomes are living or not, inasmuch as these chromosomes never undergo mitosis and ultimately undergo autolysis in the pupal stage. It is felt, however, that the most rigorous approach in this type of study is one which must involve maintenance of the general appearance and properties of the chromosomes characterizing those in the freshly dissected gland. Precautionary methods, shortly to be described, were established, therefore, to preserve this fresh state through the period of observation. The experiments performed fall into two categories, those dealing with chromosomes in the intact cell, and those on isolated chromosomes.

The Intact Cell

In studies on the chromosomes in the intact cell, the salivary gland was quickly dissected from the larva into isotonic Ringer's solution and transferred immediately to a fresh drop of Ringer's on a coverslip, which was then mounted on the moist chamber of the Chambers micromanipulator. Two precautions were necessary in such a dissection: (1) to prevent the gland from coming in contact with torn larval tissues; and (2) to remove the gland immediately from the original medium, which becomes progressively more acid in the presence of injured tissues. When these precautions were taken, the chromosomes were always distinctly visible, with relatively little space between them. The bands were generally well defined and were either homogeneous or beaded, the beads going through the chromosome. Occasionally, delicate longitudinal striations were visible in the interband regions, although these structures were usually homogeneous.

Observations and micromanipulation experiments were routinely made with a Leitz microscope using a 1.8 mm. oil immersion objective, N.A. 1.25, and a 10 X ocular. A micrometer ocular was used for measurements.

Tearing the cytoplasm with a microneedle caused marked changes in both the nucleus and the chromosomes. The nucleus swelled about 50 per cent in diameter, and the chromosomes became dark, shrunken, and so sticky as to be unmanageable. These changes appeared to be in part, at least, the result of "acid of injury," since they could be duplicated by immersion of the gland in an acidic medium. Puncturing the cell with a large microneedle produced effects essentially similar to those caused by tearing the cytoplasm. By using a graded series of microneedles, however, it was found that a microneedle of less than 1 μ toward the tip produced no visible change in the nucleus or chromosomes. Consequently, in all my experiments these fine microneedles were used.

Stickiness. The question of whether or not the chromosomes are sticky has often been raised in the literature. Practically all investigators who have manipulated the isolated chromosomes report them to be highly sticky. The fact remains that chromosomes within the intact nucleus do not appear to adhere to each other. The following experiment was done to investigate this point.

Two microneedles were inserted into the nucleus and manipulated in such

a way as to force two chromosomes together. Chromosomes approximated in this manner never stuck together. They appeared always to be separated by a narrow region of hyaline material representing the jelly-like nuclear matrix.[16] Oil drops or carbon particles which were injected into the nucleus as close as possible to a chromosome failed to adhere to the chromosome, as might be expected if the chromosomes are actually non-sticky. On the other hand, when the cell was torn, the chromosomes readily stuck together and could be separated only by pulling out thick, viscid strands. Injected oil drops or carbon particles now readily adhered to these chromosomes. It would appear, thus, that chromosomes in the intact nucleus become sticky by experimental treatment and it is likely that this phenomenon involves liquefaction of the nuclear matrix which normally keeps the chromosomes separated.

Consistency. Experiments on the consistency of the chromosomes showed them to be almost fluid in the intact uninjured cell. They were easily deformable by even slight pressure from the microneedle. Of course, any measurement on the consistency of chromosomes must of necessity be relative, and at best only rough estimates can be made. The relatively low consistency of the intact chromosome, as compared to other nuclear structures and cytoplasm, could be demonstrated by the injection of oil drops. When an oil drop was injected directly into the jelly nuclear matrix, a rounded depression was produced in the nearest chromosome, indicating that the consistency of the chromosome is less than that of the nuclear matrix. The consistency of the chromosome, on the other hand, appears to be greater than that of the nucleolus. This was demonstrated by separating the nucleolus from the small fourth chromosome (to which it is normally attached), whereupon the nucleolus became spherical, and then approximating the free spherical nucleolus to a chromosome. When this was done, the nucleolus assumed the contours of the chromosome, thus demonstrating its lower consistency. Differences in consistency could be demonstrated within the chromosome itself. Rough estimates of the relative consistency of the band and interband regions were made by determining the rate at which a micropuncture would disappear. A puncture made in the nucleolus closed over most rapidly, one in the interband regions less so, and one made in a thick band most slowly of all. It seems from these experiments, then, that the consistency of the chromosome as a whole lies between that of the nucleolus and the jelly-like nuclear matrix, and that within the chromosome the bands have a greater consistency than the interbands.

Extensibility and Elasticity. Attempts were made to determine the extensibility and elasticity of the chromosome within the nucleus by inserting two microneedles into a short section of a chromosome, then slowly drawing them apart. When this was done, the region under tension became fainter as the chromosome was stretched. The interband regions stretched more readily than the bands and consequently suffered a greater decrease in diameter. The thick bands stretched more at their margins than in the center. With increased stretching, these thick bands often separated into several narrow bands, thus demonstrating their compound nature. Faint longitudinal striations, which followed the lines of tension, generally

appeared in the interband regions when a chromosome was stretched more than twice its length. They did not appear to be identical with the delicate longitudinal striations occasionally seen in unstretched fresh chromosomes. On release of tension, the striations disappeared and the chromosome, even after being stretched up to five times its length, returned to its original condition. It was not found possible to stretch a given region more than this without tearing the nuclear membrane. More extensive experiments will be considered in the section on the isolated chromosomes.

The Chromosome Membrane. There has been much controversy as to whether or not the chromosome possesses a membrane or sheath. Painter originally thought a membrane was present, but more recently[17] has reversed his opinion. Metz[18] believes a sheath to be necessary on theoretical grounds. The experimental evidence which follows is in accord with the presence of a membrane.

A microneedle was inserted into a margin of a chromosome and then gently withdrawn a short distance. Attached to the needle could be seen a delicate membrane-like material (FIGURE 1,a).* This area was then injected with

FIGURE 1.(a) Lifting by a microneedle of a membrane-like material from a portion of a chromosome within the intact cell. (b) Micro-injection of a carbon suspension into the area subjacent to the lifted membrane shown in (a) (c) Concentration of the carbon particles into a narrow zone as a result of gradual contraction of the membrane.

an aqueous suspension of carbon particles. The material suspected of being membranous in nature became distended (FIGURE 1,b). As the fluid was dissipated, the carbon particles became concentrated close to the chromosome (FIGURE 1,c). When larger amounts of fluid were used, the membranous material would suddenly burst, with the carbon particles scattering into the nuclear matrix. The experiment was repeated by injecting an oil drop instead of carbon particles. In this instance, the oil drop became attached to the chromosome by the membrane-like material previously described and movements of either one with the microneedle would carry along the other structure.

The effects of injecting water or salt solutions (0.01–1.0 M NaCl, KCl, and $CaCl_2$) directly into a chromosome are indicated in FIGURE 2. In each instance, the bands disappeared in the region injected as the site gradually swelled, with the swelling eventually becoming localized by persisting heavy bands. If the swelling exceeded about three times the diameter of the chromosome, then the region would suddenly burst, indicating an elastic membrane whose limits had been exceeded. It seemed particularly significant

* This figure and all subsequent ones have been previously published.[21]

that the bands would, in a short time, reappear in their original positions, but the distinct outer boundary of the chromosome was lacking in the injected region. When the injected solution was calcium chloride, a granulation appeared in the nuclear matrix around the injected zone, but this did not occur with the other solutions. It would seem from these experiments that the salivary chromosome possesses a delicate elastic membrane, and that there must be structural continuity between the bands and the interband regions, since they maintain their orientation even in the absence of the membrane.

The State of the Chromosomes in Nuclei Rendered Optically Homogeneous by Experimental Treatment. The reappearance of the bands in the experiments just described suggested the possibility that in optically homogeneous nuclei the chromosomes, though not visible, are really present. Hyaline nuclei were not found in the *Chironomus* glands examined, but they could be readily made so by immersing the gland in hypotonic or alkaline Ringer's solution.

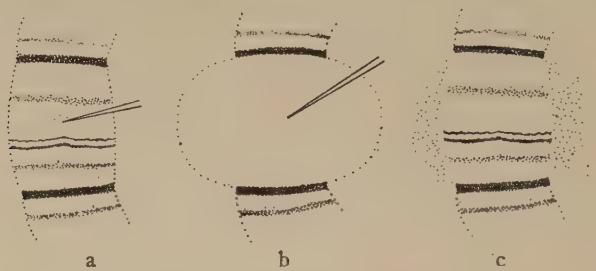

FIGURE 2.(a) Micropipette inserted into an interband region of a portion of a chromosome showing the initial stage of injection. (b) Localized swelling and loss of structure in the injected portion of the chromosome. (c) The appearance of the same chromosome after injection of sufficient $CaCl_2$ to rupture the injected region. The bands have reappeared in their original positions, but the distinct boundary shown in (a) is lacking. Note the granular region of the nuclear matrix.

Microneedles were moved back and forth in such hyaline nuclei in an effort to detect some regional resistance which might indicate the presence of chromosomes, but there was none. Stefanelli[9] made the same observation and concluded that the chromosomes must therefore be completely dispersed. Such an experiment is not conclusive, however. To test this further, the following experiment was done. Carbon particles were introduced into both normal and hyaline nuclei. The carbon particles in normal nuclei aligned themselves in such a fashion as to outline the contours of the chromosomes. When the gland so injected was subsequently immersed in hypotonic or alkaline Ringer's solution, the chromosomes swelled and disappeared, but the carbon particles maintained their positions, outlining the ghost chromosome cylinders (FIGURE 3). Similar results were obtained in nuclei which were first made hyaline, then injected with the carbon suspension. In both cases, the chromosomes reappeared in the expected positions when the glands were placed in Ringer's solution. These results suggest that the chromosomes do not become dispersed in optically homogeneous nuclei, but that they maintain their morphological integrity.

Chromosome Structure. Many experiments were made to determine the finer structure of the chromosomes. As noted previously, the detailed structure was not generally visible in the fresh chromosome, although the bands were occasionally beaded and the interband regions occasionally showed delicate longitudinal striations. These striations were visible at all levels in the chromosome and appeared to be connected to the beads in the bands. An alveolar appearance was never observed in the fresh chromosome, but the alveolar condition was seen often in aceto-carmine or Feulgen preparations.

Chromosomes were treated with various reagents in an attempt to dissolve differentially the chromosome so that its basic structure would be more clearly revealed. Results of this type are valuable but must be interpreted with caution, since it was found possible by experimental treatment to cause the chromosome to appear fibrous (with alkali[17, 19] or alkaline urea[4]), alveolar (with NaCl,[17] KCl, or $CaCl_2$, the latter only if injected), granular (with

FIGURE 3. Alignment of carbon particles outlining the contiguous borders of adjacent chromosomes in an optically homogeneous nucleus. The nucleus was first injected with a carbon suspension, then hyalinized by immersion of the gland in hypotonic Ringer's solution.

NaCl,[17] KCl, acids, or detergents), or homogeneous (with organic solvents). Accordingly, recourse was had to other methods.

If chromonemata are really present, it should be possible to obtain evidence from micrurgical experiments. These were done as follows. Oil drops were injected into the interband regions of the chromosomes to determine whether the oil might possibly become elliptical in shape as a result of compression between alleged chromonemata. The injected oil drops were, however, always spherical. This would tend to support the alveolar contention of Metz and Lawrence[1] or Hinton's concept[6] of structureless interbands. But it does not necessarily invalidate those concepts which involve the presence of chromonemata, since it will be remembered that the chromosomes have a very low consistency and therefore existing chromonemata, if any, could easily be displaced.

Attempts were made to obtain evidence from lateral stretching experiments, since there was the possibility that any existing chromonemata might be separated in this way. Two microneedles were inserted into the margins

of a chromosome and then slowly pulled apart. It was observed that the band, being stretched, became beaded (FIGURE 4). This might be taken to indicate that the chromomeres were being separated from each other. (A similar beaded appearance was observed when the nucleus was injected with water or dilute salt solutions.) The interband regions showed no chromonemata, but tension lines were visible.

More clear-cut evidence was obtained in microdissection experiments. The chromosome membrane was first removed over a short distance. Then a microneedle was inserted close to the edge of the chromosome. It was found possible to remove delicate longitudinal fibrils in this manner, even though no striations were visible in the region being dissected. The fibrils thus partially isolated displayed nodes at intervals corresponding to the position of the bands (FIGURE 5). These nodes did not disappear when the fibrils were stretched, indicating that they are not merely gyres in a coil as

FIGURE 4.(a) Sketch of a portion of a chromosome prior to stretching. Two microneedles are inserted directly into a band. (b) Beaded appearance of the band when the chromosome is stretched laterally. Note tension lines in the interband regions.

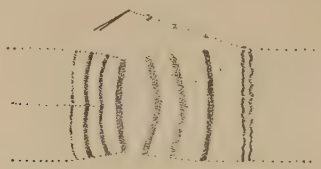

FIGURE 5. A single fibril with a chromonema-like appearance being removed by a microneedle from a chromosome within the intact cell.

maintained by Ris and Crouse.[5] Similar fibrils could be obtained by injecting a small amount of fluid near the margin of a chromosome. When the injected fluid was calcium chloride, the fibrils were stiff and inelastic. On the other hand, fibrils isolated by the injection of water, NaCl, or KCl, were soft and highly extensible. It was not found possible to dissect any fibrils lying at right angles to the long axis of the chromosome, as should be equally possible in an alveolar structure. This evidence, as well as that shortly to be described from the isolated chromosomes, appears to support best the polytene concept of chromosome structure.

Isolated Chromosomes

The most important factors in the isolation of a single salivary gland chromosome are: (1) immediate removal of the chromosome from the torn

cell; and (2) quick transfer of the chromosome to a neutral calcium-free medium. In these isolation experiments, the injurious changes produced by the surrounding cytoplasm on the nucleus were minimized by rapidly tearing both the cytoplasm and the nuclear membrane. The operation was done with steel needles under a dissecting microscope. In this way, the chromosomes could be forced out of the nucleus by gentle pressure, quickly picked up by a lip pipette, and then transferred to the appropriate medium. No exhaustive attempts were made to find the ideal medium for the isolated chromosome, but of the various media tried, including hemolymph, Ringer's, paraffin oil, sucrose, albumen, Duryee's medium,[15] and 0.6% NaCl, the most satisfactory medium was one consisting of 0.09 M KCl, 0.06 M NaCl, buffered to pH 7.0 with phospate. In this medium, the isolated chromosomes closely resembled those in the intact cell in both structure and physical properties for at least 5–10 minutes. All observations were made within this period.

It was found that relatively small changes in pH could cause extensive changes in the structure and properties of the isolated chromosomes. If the chromosomes were maintained at pH 7.6, reported by Chambers[20] to be the pH of the normal *Chironomus* nucleus, structure became faint, and the chromosomes became sticky and difficult to manipulate. Acidification of the medium to pH 6.5 caused the chromosomes to shrink, particularly in the interband regions, and structure to become more distinct. Such chromosomes were relatively stiff and inelastic. The consistency of the chromosomes increased progressively as the pH of the medium was lowered. The effect of calcium ions on the isolated chromosomes was generally like that of acidification.

The isolated chromosomes were especially satisfactory for stretching experiments. Both extensibility and elasticity were influenced considerably by the medium. Acidic solutions decreased both properties, whereas, in alkaline solutions, extensibility was greatly increased, the chromosomes becoming ductile and inelastic. In the neutral KCl-NaCl medium, chromosomes could be stretched about 10-fold and still recover their original length, although repeated stretching tended to decrease elasticity. In one case, a chromosome was stretched 25-fold, and when tension was released, it returned to slightly more than its original length (1.3x). This observation, perhaps, is significant in view of the established fact that long fibrous molecules possess unusual elasticity. When rupture occurred in one of these stretched chromosomes, the break invariably occurred in an interband region. Fibrillae were occasionally seen at the broken ends of the chromosomes. It should be pointed out that greatly stretched chromosomes developed a coarse fibrillar appearance and rapidly deteriorated. This fibrillar appearance was different from that observed in unstretched chromosomes in that these fibrils were relatively coarse and always followed the lines of tension.

I believe, however, that the following experiment indicates that true longitudinal fibrils do exist in the isolated chromosomes. It was possible to remove a whole chromosome directly from the nucleus by means of a very large micropipette. It was further possible to expel the chromosome from

the pipette into an appropriate medium. If the micropipette was of the correct caliber, the expelled chromosome would shred into numerous delicate longitudinal fibrillae which resembled the fibrils obtained by microdissection. The operation was only occasionally successful, for, if the micropipette was much larger than the chromosome, the chromosome would be expelled intact. The fact that this shredding of the chromosome into longitudinal fibrils can occur, however, is deemed significant, and this observation—together with the beading of the bands, the dissection of fibrils by microneedles, the occasional fibrillar appearance of untreated chromosomes, the presence of fibrillae at the broken ends of stretched chromosomes, and the return of the bands to their original positions after rupture of the chromosome membrane—tends to show the polytene nature of the salivary gland chromosome.

In summary, it may be concluded, from these micromanipulation studies, that: (1) the salivary gland chromosomes are highly extensible, elastic, soft, deformable bodies, and not viscid, tough gels as generally described by others; (2) the salivary gland chromosomes in optically homogeneous nuclei are not isolated or dispersed, but rather maintain their morphological integrity; (3) the salivary gland chromosome possesses a delicate elastic membrane; (4) the salivary gland chromosome is polytene in nature.

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Discussion

Ivor Cornman (*The Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): Such superbly delicate probing into the chromosome is supplying us with the answers to questions which remained controversial the whole time that we were forced to rely upon indirect evidence of chromosome structure. Is the matrix which separates chromosomes, as made visible when the chromosomes are pushed together, to be visualized as a sheath around each chromosome? Can it be distinguished from the chromosomal membrane?

In a hyalinized nucleus, where the boundaries of the chromosomes are marked with carbon particles, can the chromosomes be moved by the microneedle? If they are so fluid as to permit the needle to pass through them, how does a chromosome look when again rendered visible?

Can it be demonstrated that fibrils dissected from the giant chromosomes behave in a manner comparable to chromonemata, as for instance, spiralling in acid medium?

Doctor D'Angelo: It may be shown that the chromosomes in salivary-gland nuclei are embedded in a jelly-like matrix by various micrurgical experiments. Carbon particles injected into the central region of the nucleus show no Brownian movement, whereas particles injected just under the nuclear membrane display Brownian movement. An oil drop injected into the central region of the nucleus remains in position, whereas one injected peripherally will tend to rise as would be expected in a fluid medium. The jelly-like matrix is thixotropic, as it may be liquified by mechanical agitation with the microneedle. The chromosome membrane is still demonstrable after liquefaction of the matrix has occurred.

Chromosomes outlined by carbon particles in hyaline nuclei may be moved about by microneedles, and are sufficiently fluid so that the needle may pass through them. When such a nucleus is returned to Ringer's solution the chromosomes appear distorted.

Further experiments must be performed before Dr. Cornman's last question can be answered.

CHROMOSOMAL PHYSIOLOGY IN RELATION TO NUCLEAR STRUCTURE

By William R. Duryee

National Cancer Institute, Bethesda, Maryland, and Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D.C.*

Statement of the Problem

In every cell nucleus, there exists a structural as well as a physiological relationship between the chromosomes and the colloidal substrate in which they are embedded. Not only does the substrate maintain and protect the chromosomes in their relative positions, but it also transmits to them organic and inorganic molecules for synthesis. Even more important, the substrate collects and distributes the materials produced by the chromosomes. The basic significance for these processes in amphibian eggs of lateral loop synthesis was first presented by the author in 1937, followed by a general theory of the vertebrate chromosome (1941).

The periodic disappearance and reappearance of the lateral loop chromomere structures have led some, through fragmentary observations, to deny their existence. For example, Clark, Barnes, and Baylor (1942), using the electron microscope found no evidence for them. On the other hand, Boche and Anderson† saw them clearly and figured them in their electron photograph. Lateral branches of amphibian chromosomes were first described by Flemming (1882), while the loop structure was discovered independently by Born (1892), in amphibia, and Rückert, in sharks, in 1892. Studies by M. Gersch (1940), Painter (1940), Painter and Taylor (1942), Ris (1945), and Koltzoff (1938) on amphibian material have failed to show how chromosomes function. They disagree on the basic details of lateral loop structure and on results of Feulgen staining, as well as on the significance of nucleolar production. A more serious error has recently been made by P. Makarov (1946), who claims that *Rana temporaria* ovarian chromosomes are experimentally created "*de novo*" by fixatives penetrating the "homogeneous nuclear colloid." He did not attempt to account for constant chromosome numbers or specifically recognizable chromosome pairs. With the exception of Gersch's paper, which concentrated mainly on chemical tests of nucleoli, there is no adequate work on the physiology of animal chromosomes. Dodson (1948) has studied the Feulgen staining of amphibian chromosomes and is in general agreement with the morphological results reported here.

The main purpose of this paper is to show experimentally how chromosomal products are liberated from the chromosomes within the cell nucleus and transmitted to the cytoplasm. A secondary purpose is to present a new technique that is based on photographic records of fresh material under precisely controlled conditions. The older techniques of fixation and staining have led to no new point of view. Precipitation of colloidal proteins with harsh reagents, followed by long extraction in fat solvents and exposure

* This work was done at the Marine Biological Laboratory, Woods Hole, Mass., and at New York University in the laboratory of Dr. Robert Chambers, to whom grateful acknowledgment is made herewith.

† See review by G. A. Morton.

to hot wax and aniline dyes, cannot be considered a physiological method. From a detailed study of alternately mounted sections of testis and ovary fixed in Zenker, Carnoy, Bouin, Aoyama, and other fluids, it is concluded that, while the spermatocyte material gives the ideal conventional but doubtless erroneous pictures, fixed ovocyte details differ from the normal *in every respect*. Moreover, an appreciation of correct geometry is essential to the study of the nucleus, and this cannot be obtained from dehydrated and sectioned material. One must always bear in mind that living processes can only take place in water-rich colloids (*cf.* Duryee 1938). The conclusions presented in this paper apply equally to both frog and salamander material except where specific differences are noted.

A method whereby the above difficulties could be circumvented was introduced by the author in 1936 for studying the relatively large ovocyte nuclei of aquatic vertebrates. It consisted essentially of slitting the ovarian egg to extrude the nucleus into a modified calcium-free Ringer solution.* When washed immediately from adherent yolk and cytoplasm, the isolated nucleus clearly exhibited its chromosomes, nucleoli, and other constituents in their three-dimensional relationships. The nuclear membrane could then be dissected by hand, using glass needles, thus freeing the inner colloid gels with their 13 pairs of chromosomes and the multiple nucleoli, all of which were easily available for further study with reagents or for micromanipulation.

Isolated nuclei are not necessarily alive, nor are they to be considered dead, unless toxic reagents are added. The important fact is that they appeared normal in all essentials for periods up to one-half hour isolation, when recognizable degenerative changes may occur. The mild pH changes used in this technique for photography were almost completely reversible. PLATE 1 (A) gives a comparison of transparent eggs with their visible nuclei with one that has been isolated and floated into the field.

Normal Nuclear Growth Cycle

FIGURE 1 has been prepared to show, diagrammatically, the chief stages of normal germinal vesicle development and to indicate the concurrent changes in chromosome structure. It is convenient to divide the nuclear history into six arbitrary stages. These are typical of the frog, but with minor amendments they also apply to urodeles. Stage 1 is the smallest follicle in which chromosomes can be seen in the intact and transparent living egg, but without clear definition. In Stage 2, the chromosome pairs are now barely visible embedded in a nucleoplasmic gel, when the medium is slightly acidified below pH 5.8. Egg diameters are less than 200 μ. Comparison should be made with PLATE 1 (A). By Stage 3, where the eggs are from 200 to 500 μ in diameter, a few more details of nuclear structure can be observed through the surrounding transparent theca cells. Lateral loop production has now begun. A zone of large irregular nucleoli, prominent just beneath the nuclear membrane, characterizes this stage. Shadows of

* Nuclear or N-Medium is made up of NaCl 0.66 gm., KCl 0.014 gm. in 100 cc. of Pyrex distilled H_2O. This medium should not be buffered, except in cases where nuclear swelling is troublesome. In rare cases with small nuclei, it may be helpful to reduce the pH to 5.9 with KH_2PO_4.

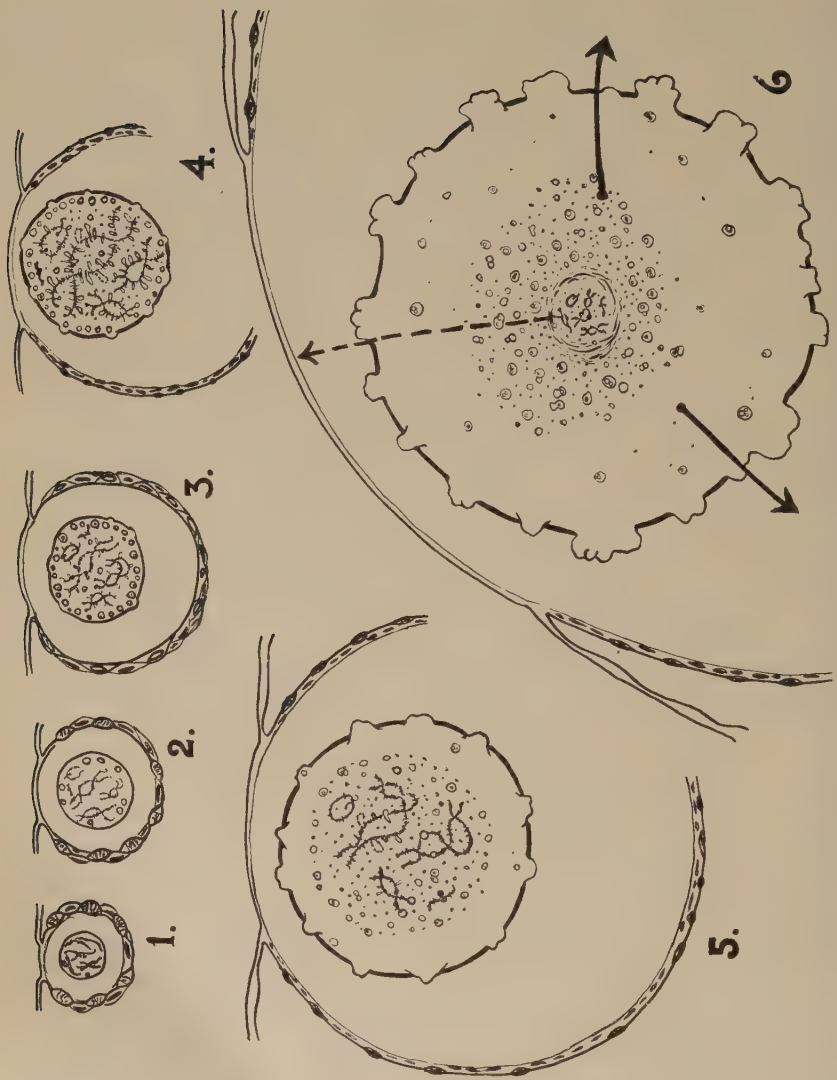

FIGURE 1. Schematic diagram of nuclear growth stages during the later development of frog eggs. Heavy arrows indicate mixing of nuclear material in cytoplasm after germinal vesicle membrane breakdown. Dotted arrow indicates migration of central chromosomal mass toward the animal pole to become the 1st polar body maturation spindle.

the paired chromosomes are barely visible under ordinary light, but are clearer with the phase contrast objective or in less alkaline media. See PLATE 1 (B). In Stage 4, the development of a yellow-brown color and addition of yolk prevent internal observation. Consequently isolation and washing of the nuclei are necessary. Eggs in Stage 4 range from 500 to 750 μ in diameter. Here the chromosomes of *Triturus pyrrhogaster* reach their maximum length of over 700 μ and have the most developed lateral branches. Frog chromosomes of comparable stages are approximately 60 per cent as large as those of urodeles. It is of interest that these Stage 4 amphibian chromosomes are the largest yet known in any animal, being nearly three times the size of the so-called giant chromosomes of dipteran salivary glands.

Up through Stage 4, growth of the supporting colloid gel, which I have designated the *chromosome frame*, keeps pace with the increasing diameter of the nucleus. This period of growth is shown in the top four diagrams of FIGURE 1. Photographs of Stage 4 before and after staining appear in PLATES 1 (C) and 2 (C).

By Stage 5, the chromosome frame begins its slow process of contraction. Eggs have now reached approximately half their maximum normal size, having diameters ranging from 750 to 850 μ. The contraction is not at first evident, since the nucleus as a whole continues to increase in volume, but becomes marked in comparative measurements made on older cells. Concurrently, the chromosomes are shortened and have progressively fewer and smaller lateral loops. Major nucleolar production is continued, as shown by new nucleoli appearing near the center and by an overall increase in number up to several hundred. Sac-like protrusions, which were first described by Duryee in 1937, are beginning to project from the surface of the nuclear membrane. Since these are formed structures of the membrane, remaining without change even in torn isolated membrane sections, the sacs may be spoken of as "organelles." In some species, they persist during a year or more of ovarian existence.

Stage 6, which is the last depicted, shows the germinal vesicle at its maximum growth, when the egg diameter has reached 1.8 mm. The diagram should be compared with PLATE 1 (E), which shows a nucleus from a somewhat smaller egg. The former indicates that the chromosome frame (= Karyosome) has shrunk to less than 1/1000th of the total nuclear volume, and is now coated by a denser substance, which has the property of being coagulated by calcium into aster-like fibers. The chromosomes have shortened to 40 μ or less and have lost all large lateral loops. A striking feature of this stage is the central cloud of large polyphasic nucleoli, surrounded by a few peripheral ones. Mixed with the central nucleoli are a number of distinctly smaller hyaline bodies having diameters from 0.5 to 2.0 μ. These latter may be called *loop fragments*. Together with the larger nucleoli they are embedded in a physically separable gel concentric with the chromosome frame. The fact that chromosome frame substance is a morphological entity in the nucleus can be seen in PLATE 1 (D). Here the other supporting gels have been dissolved, allowing the chromosome frame to sink

to the inside of the nuclear membrane as a spherule. Its viscous nature is shown in PLATE 1 (F).

As has long been known, the history of the egg nucleus terminates with a process of dissolution of the membrane and a belated intermingling of

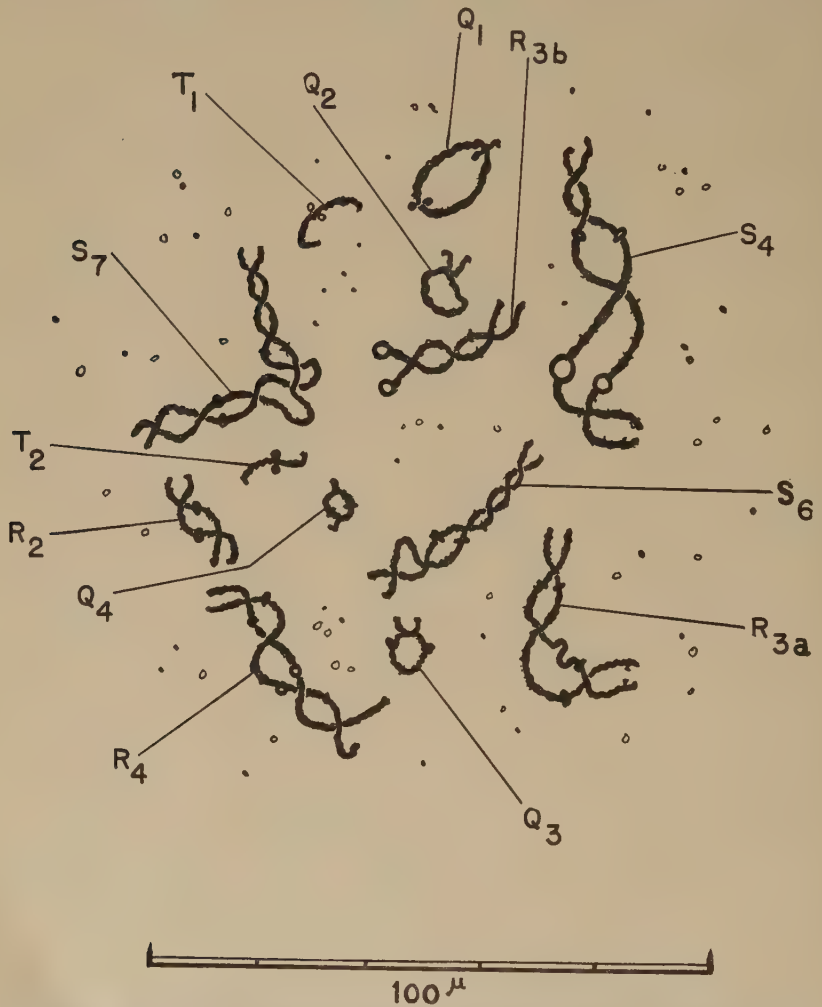

FIGURE 2. Camera lucida sketch of complete chromosome set in the germinal vesicle of a Stage 6 frog egg. Identifying letters have the following significance: *Q* for ring or "Q" -shaped pairs (4); *R* for intermediate pairs (4); *S* for long or super pairs (3); and *T* for short "T"-shaped pairs (2). Subscript numbers help the identification of the *R* and *S* group by indicating the number of chiasmata which is constant for a given chromosome pair. In the case of *T* and *Q* sets, they indicate merely decreasing size.

germinal vesicle contents with the egg cytoplasm. This process initiates a profound metabolic reaction which exhausts the cell unless fertilization shortly ensues. At the same time, the chromosome frame, having been set free, is converted into the 1st maturation spindle preparatory to extrusion of the polar bodies [*cf.* FIGURE 1(6)].

Identification of Isolated Chromosomes

FIGURE 2, a camera lucida sketch, shows the relative shapes and sizes of the 13 pairs of chromosomes in a Stage 6 nucleus of *Rana temporaria*. From comparison of egg nuclei in the following species, *R. temporaria*, *R. esculenta*, *R. pipiens*, *R. catesbiana*, and *R. clamitans*, it is clear that the numbers and individual characteristics of the chromosomal pairs are remarkably constant. This leads to an advantage for studies of chromosome physiology, because each individual pair can be precisely identified. For convenience, they have been designated with the letters *Q*, *R*, *S*, and *T*. Shown in FIGURE 2 are four ring or Q-shaped pairs identified by the letter *Q* and subscript numbers in order of decreasing size. Next come four medium pairs lettered *R* with numbers corresponding to the number of chiasmata. *S* designates the three longest or super pairs, again with subscripts referring to the number of chiasmata. The letter *T* identifies the two short T-shaped pairs. Sufficient data are not yet at hand to permit a similar chromosome identification system for the other groups of amphibia.

Basic Chromosomal Structure

The amphibian chromosome, because of its size, permits an analysis of structural elements not heretofore possible. Much of the confusion that exists regarding interpretation of stained and fixed material can be avoided by the study and photographic recording of freshly isolated nuclear components. The details are sharp and clear. In this section, the basic elements of chromosomal structure are presented, followed by the experimental and analytical data. First to be described will be those chromosomes without lateral loops.

FIGURE 3 illustrates the fact that both frog and salamander chromosomes have a similar structure. There is an apparently single chromonema along which compound granules and chromomeres of varying shape and size are firmly embedded or attached. Both pairs of chromosomes were in normal intra-nuclear position when sketched. The salamander pair, being under slight tension except for the left-hand end of one univalent, shows the general correspondence between homologous chromomeres. At certain places, however, a few of the visible structures do not appear to correspond. In chromosomes such as these, which have been treated with 0.2 M. $NaHCO_3$ to remove the lateral loops and reveal the chromonemata, there is no evidence of uncoiling or breakage of longitudinal elements. Occasional loops may still persist after this mild treatment showing they are not part of the chromonemata. Photographs in PLATES 2 (A), 5 (C, D, E, and F) and 6 (A and B) illustrate the same details. The thicker oblong areas on these chromosomes are gelatinous or sometimes viscous coatings of the longitudinal chromonemata. Since this sheath-like coating is similar to that described for other types of plants and animals by various authors, *e.g.* Nebel (1939), as the chromosome "matrix," that term will be retained here. It is not so easy to homologize the terms "chromomere" or "chromiole" with the structures in FIGURE 3. By definition, a chromiole is the smallest visible particle,

constant in size, that remains embedded in a chromosome. Until the chemistry of these particles is better known, I prefer the term "chomosome granule" to refer to such particles 1 μ in diameter or less. "Chromomere" is the name correctly applied to an individual discrete segment of somewhat larger size, together with its cluster of lateral branches described below. As far as can be seen in the accompanying photographs, there are seldom more than two granules per chromomere. However, it is possible that adjacent chromomeres may fuse, as shown in PLATES 5 (C) and 6 (B).

The second type of basic structure is that of the Stage 4 and 5 chromosomes with large lateral chromomere loops. FIGURE 4 is a diagram of the loop cluster arrangement in normal nuclei at different stages. This figure should be compared with PLATES 2 (B, C), 3 (E), and 4 (A, B, C). In the diagram at the left of FIGURE 4, several clusters of lateral loops (1) are shown

FIGURE 3. Camera lucida sketch of Stage 4 chromosomes. Short exposure to 0.2M $NaHCO_3$ followed by mild acidification has removed most of the lateral loops. Chromosome granules are attached to the paired chromonemata at homologous loci. Some matrix coating is present.

held together by a single chromonema (*k*). Attention is called to two other important points, namely, that there are different numbers of loops per cluster, and that loops are not all of the same construction. At the right of the same figure is a diagram of a chromosome from a Stage 5 or early Stage 6 nucleus. Most of the large loops have disappeared, leaving an accumulation of loop fragments. The few that remain often show partly straightened broken branches, which in no way affect the chromonemata. The granules and the chromonema have, in certain areas, acquired coatings of matrix material (*m*). This is a weak gelatinous coating not to be confused with the longitudinal thread.

Chromosome Formulae

By use of the letters shown in FIGURE 4 and explained in the previous paragraph, a convenient system of formulae has been made. The annotation shows what basic units of the chromosome are present. For example, FIGURE 4 shows a *k l m g* type chromosome, since all four structural units are markedly developed. On the other hand, FIGURE 2 shows *k m g* types, the

standard for later stages. In FIGURE 3, the frog chromosomes are *k l m g*, while the salamander pair are *k m g* types. The value of this system may be seen where large numbers of chromosome photographs need be compared and where homologies between widely differing species of animals and plants are sought. Thus, the *Chironomus* salivary gland chromosome, described so well by Dr. Ethel Glancy D'Angelo (1946), would be of the *k m g* type. Tissue cell chromosomes in mitosis appear as *k m* types, since the granules

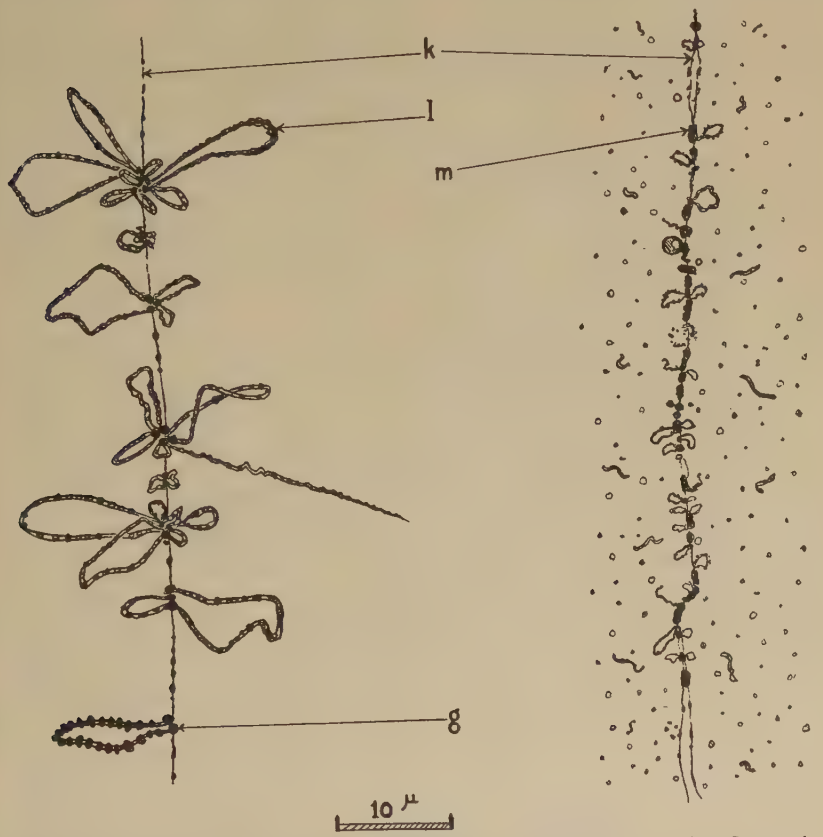

FIGURE 4. Diagram of normal amphibian egg chromosome structure at Stages 4 and 5. Stage 4 chromosome at left consists of lateral loop (*l*) clusters of varying type and numbers held together by an apparently single chromonema (*k*). Loops originate from paired granules (*g*). Stage 5 chromosome at right shows many loop fragments and few residual broken and disintegrating loops. Matrix material (*m*) coats the chromonema, which only rarely shows evidence of a split.

are not visible. Chromosomes in the male germ cells of amphibia, which normally pair with those described in this paper, have also been observed as *k m* types. But it should be emphasized that all *egg* chromosomes brought to their isoelectric point of pH 4.5 (Duryee 1941) or stained with basic dyes show there are granules present. Chromosome formulae may easily be determined from rapid smear preparations such as illustrated in PLATE 2 (A).

The following tabulation gives the formulae for the chromosomes in the accompanying photographs:

Plate number	*Chromosome formula*
2 (A)	*k m g*
(B)	*k l g*
(C)	*k l g*
3 (E)	*k l m g*
4 (A)	*k l g*
(B)	*k l g*
(C)	*k l m g*
5 (A)	*k m g*
(B)	*k g*
(C)	*k m g*
(D)	*k m g*
(E)	*k m g*
(F)	*k g*
6 (A)	*k l m g*
(B)	*k m g*

Chromosomal Function

The cytological evidence analyzed in this section leads to two general conclusions. One is that the germinal vesicle chromosomes control the synthesis of new material in the form of lateral loops, which grow outward and then dissolve into a host of barely visible particles—the loop fragments. The second is that nucleoli of larger size are produced at definite loci and, likewise, accumulate in the nucleoplasm. Both of these ideas have long been current. Indeed, Rückert, in his classic paper, clearly stated that the side loops of *Pristiurus* chromosomes are converted into nuclear granules. However, no experimental analysis of this important problem in germ cells has been made. The present discussion will be limited to physical studies on chromosomes in known chemical media. For more strictly chemical data on amphibian germinal vesicles, the reader is referred to the paper by Gersch, who used my isolation techniques and media.

Loop Production and Variations

Lateral loops begin to grow outward in Stage 3 nuclei and reach their maximum in Stage 4. The base of each loop always appeared associated with definite granules in the *k* thread, which stained more intensely than loop material. Attention is directed to PLATE 2 (B and C). The originating granules within each of the small clusters are clearly shown. There is no trace of loop material in the spaces between clusters. By the simple expedient of removing the nuclear membrane and adding a drop of 0.1 M. NaH_2PO_4, especially clear observation was possible. No case of "coiling" from one cluster to the next has ever been noted.

Loops varied greatly in size, not only between different clusters but also in the same cluster. Figures reported here are measurements made on chromosomes at the isoelectric point, while the ocular micrometer scale was

read critically to one micron plus or minus 0.5 at a magnification of 1600 X. On freshly isolated material, the measurements are correspondingly from 5 to 20 per cent larger, but were more subject to error on account of lack of definition. In frog material, Stage 4 chromosomes in a single nucleus had loops projecting 2.5 to 24 μ from the *k* thread. Average lateral projection was 9.5 μ. Urodele structures were relatively much larger. For example, the average lateral extension of loops in *Triturus pyrrhogaster* was close to 15 μ with extremes of 2 to 36 μ. PLATE 2 (B) shows a typical mixture of large and small loops in a single cluster. The photograph also brings out the fact that the number of loops per cluster varies from one to nine. Adjacent clusters on the same chromosome usually have different numbers of loops. This is also apparent in PLATE 4 (A).

The structure of each individual loop is of great importance. FIGURE 4 and PLATES 2 (B) and 4 (B) show that the loop consists of a hyaline cylinder approximately one micron in diameter. Embedded in this cylinder are the denser particles averaging 1.5 μ whose fate is discussed in a following section on "Loop Transformation." They are clearly not optical sections of "minor coils." When freed from the chromosome frame gel, as shown in PLATE 4 (A and B), the cylinder will contract into wavy lines, but the particles are still observable *in situ* and do not correspond with the irregular angles thus formed. Addition of standard fixatives or coagulating reagents produced and accentuated such artifacts, but the embedded particles remained discrete bodies.

Not all the loops on a single chromosome were alike. In *Triturus pyrrhogaster*, there always appeared one pair of chromosomes, each having a single unusual lateral loop. This was a heavily beaded structure, each bead being about 7 μ in diameter. At the corresponding loci, each loop originated from a pair of granules in the *k* thread, as indicated in FIGURE 4. The fact that loops can be different indicates that they are not parts of a continuous thread.

Loop Numbers. While it has been shown that the loop numbers vary from one unit cluster to the next and are not necessarily multiples of one another, the number of clusters per 100 μ of any chromosome in a single nucleus is remarkably constant. Some sample calculations based on the accompanying photographs will make this clear. For the purpose of comparison, a standard length of 100 μ has been selected. The chromosome in PLATE 2 (B) has approximately 23 loop clusters or centers of activity per 100 μ. Projecting from these loci are 71 loops $\pm$ 8. Allowing an additional 15 per cent for loops which do not appear, being above or below the focal plane in the photograph, it can be estimated that there are about 80 to 90 loops per 100 μ unit of length. The ratio of 80:23 shows that at Stage 4 there is a rough average of about 3.5 loops per cluster.

In more advanced stages, the average drops. This is brought out in PLATE 3 (E), which is a photograph of Stage 5 *Triturus* chromosomes. The long pair with three chiasmata measures 435 μ. Approximately 120 chromomeres or centers of activity can be counted in each chromosome. Therefore, there is an average of 28 chromomeres per unit of 100 microns, which

in comparison with Stage 4 indicates a slight shortening of the *k*-thread. On these same chromosomes, approximately 213 loops ± 14 can be counted. This gives an average of about 49 loops per 100 micron unit. The ratio of 49:28 gives an average of 1.7 loops per chromomere of cluster at Stage 5, in comparison to 3.5 at the earlier stage. By Stage 6, only a few loops could be found on the chromosomes. Subsequently, the averages become still lower, approaching zero. In summary, the number of loops per chromomere decreases with time, although the total number of chromomeres remains constant.

Loop Solubility. Lateral loops were dissolved off the chromosomes in a variety of ways without affecting the *k* thread. Solvents included dilute hydroxides (0.1 N) and bicarbonates and basic phosphates, the more hydrating anions of the Hofmeister series—such as Sulphide, Iodide, Thiocyanate and Citrate, and also KCl (0.01 M). Irradiation with X rays (over 50,000 r) and ultraviolet containing a strong band at 2537 Å also caused dissolution of the lateral loops.* The X-ray experiments have been done with many hundreds of isolated nuclei, both frog and urodele, in the middle stages. All gave the same result, namely, that loops so treated were transformed into granules. On reacidification to pH 4.5, the chromosome pairs reappeared exceptionally sharp and with the same number of chiasmata, but without lateral branches. No evidence of any resorption back into the *k*-thread has been found. PLATE 5 presents six photographs of different types of chromosomes from which the lateral loops have been removed by various means. Whether or not matrix material remained after treatment depended upon the type of ion used and on the duration of exposure.

Reagents applied directly to isolated chromosomes gave clear results. In favorable experiments using very thin films (where reagent penetration was slowed by pressing down the cover slip into the plastic cover slip supports), actual disintegration of the loops was observed directly. Particular attention was paid to the chromonemata. No breakage or significant change was observed in them. In PLATE 5 (B), a pair of *Triturus pyrrhogaster* chromosomes is shown in which the chromonemata are twisted at the chiasma. All remaining lateral loops were dissolved off by short exposure to 0.01 M KCl. PLATE 5 (C) shows a single *Triturus* chromosome after momentary treatment with 0.1 M Na_3PO_4 followed by 0.003 N HCl. Although the terminal nucleolus was preserved, the lateral loops went into solution and were converted into granular fragments. PLATE 5 (D) illustrates a "Q" chromosome from a Stage 6 *Triturus* egg. Loops have normally been dissolved away at this stage.

Extremely heavy dosages of X rays removed the loops completely from chromosomes even after they had been isolated. PLATE 5 (E) shows the effect of 100,000 r on an isolated pair in a thin film of Ca-free Ringer. It is evident that, while the *k*-thread has not been ruptured, all loops have disappeared. Dosages of 20,000 r fragmented few loops, in contrast to 50,000

* The author wishes to express thanks to Dr. C. C. Clark of New York University for loan of a convenient U. V. irradiation device. This consisted of a quartz, mercury vapor discharge tube (1 cm. diam.) bent in the form of circle. It was mounted so that the microscope objective fitted through the circle. Isolated nuclei were irradiated through quartz cover slips 1 cm. from the source for 300 seconds. Sixty seconds of irradiation produced no effect.

r, which caused complete dissolution and left the chromosomes a granular hyaline cylinder. PLATE 5 (E) also demonstrates a high degree of correspondence between the similarly situated *g* granules in each chromosome. X rays did not remove the matrix; consequently each one is seen to be of the *k m g* category.

The complete transformation of a *k l m g* type chromosome into a *k g* form is illustrated in PLATE 5 (F). Here, 0.1 M Na_3PO_4 was allowed to act for two minutes before acidification to pH 4.5. Only a central thread and some embedded granules remained, although the chiasma was still clear. The granules appeared as lumpy discrete masses. Since both Painter (1942) and Brachet (1929) have described these granules as Feulgen positive and since all their physical properties resemble the dark bands in insect salivary chromosomes, it seems possible that they are rich in desoxyribose nucleic acid. On the other hand, my own Feulgen preparations, under ordinary optics, have consistently shown all portions of Stage 4 chromosomes as negative; yet loops, granules, and chromonemata were clearly visible on switching to phase-contrast. Attention is drawn to the facts that in a chromosome pair not only is the spatial distribution of the granules along each *k*-thread a mirror image of the other, but, also, the size and shape of each granule corresponds in both homologues. As in the previous cases, no evidence of loop retraction was observed. It is concluded from the experiments typified by PLATE 5 (F) that matrix material can be completely removed leaving only the *k g* fiber.

Micromanipulation Experiments. The micromanipulative technique is well suited for many kinds of tests on chromosome function. Dissection of component parts revealed differences in physical properties. Differential solubility of loops and nucleoli were further examined by micropipetting reagents directly on the parts concerned. Chromosomes were operated with a Chambers instrument. After the nuclear membrane had been removed, the material was suspended in a hanging drop of calcium-free Ringer. PLATES 3 and 4 illustrate various types of experiments carried out.

In order to test whether the lateral loops were continuous with the longitudinal *k*-thread, a series of chromosomes were stretched in different salt media. In every case, both with anurans and with urodeles, individual loops or clusters of loops became separated from one another as units, *without the bases of the loops opening up.* No evidence of breaking of a hypothetical coil was seen even under oil immersion. Adjacent loop clusters could be separated over 30 μ until the *k*-thread could no longer be seen. On approximating the needles, *k*-threads reappeared as before and the chromosomes resumed their original appearance, matching their homologues exactly. Each stretched chromonema was found to be a single thread with slight oval bulges all of much smaller size than that of the loops. The original photograph in PLATE 4 (A) shows this condition plainly.

When matrix (*m*) substance was present, it separated on stretching into unequal segments as shown in PLATE 4 (A). It separated easily as a viscous material with no trace of snapping or breaking fibrillae, such as Ris has postulated. On approximating the microneedles, matrix segments fused or

molded together restoring the original appearance. This type of experiment is shown in PLATE 4 (C). As reported in an earlier paper (Duryee 1941), the relative elasticity of the *k*-thread varied from an average increase of +450 per cent in calcium-free media (pH 6.8) to only +85 per cent in the same medium acidified to pH 4.2. In 0.001 M $CaCl_2$, a chromosome could be stretched only about twice its length before breaking. Recent experiments have confirmed the fact that, in contrast to *k*-thread elasticity, the loop material (*l*) is always more brittle, less elastic, and more frangible. In no case could a loop segment be stretched more than 50 per cent before breaking. Such an experiment is shown in PLATE 4 (B). The conclusion is that the physical properties of loop material are markedly different from those of the *k*-thread.

Loop Transformations. It has been brought out by the foregoing sections that lateral loops originate from chromosomal granules and that the lateral branches themselves are not homogeneous in structure, but are made up of smaller particles embedded in a hyaline cylinder which has different solubility properties from the *k*-thread. This section deals with the transformations of the component particles of loop material into many separate nuclear granules or loop fragments. Reference is made to FIGURE 4. As mentioned previously, under favorable conditions with hydrating reagents added slowly and in minimal quantities, actual dissolution of the loops with liberation of embedded granules could be followed under oil immersion. The hyaline bodies so formed were identical in size, appearance, and position with those normally formed in a cell. Stage 4 chromosomes of *Triturus* given 30,000 r of X-irradiation always showed many lateral loops missing. In such places, many loop fragments were found adjacent or attached to the chromosomes. It is strongly suggested by these experiments that the characteristic normal loop fragments are produced in a similar fashion to the experimental ones.

One of the most constant normal features of freshly isolated Stages 5 and 6 nuclei was the cloud of hyaline refractive bodies seen surrounding and emerging from the central chromosome area. They are illustrated in the photographs of PLATE 6. In diameter, they measured 0.5 to 2.0 μ. Staining with basic dyes, solubility and position in the nucleus classes them with the larger nucleoli. On the other hand, constant size, refractive surface, and spherical shape differentiate them from ground-substance floccules. Loop fragments do not begin to appear until late Stage 4, when chromosome loops are beginning to disappear. They are numerous in Stage 5 and increase to between 70,000 to 100,000 by late Stage 6. It is highly significant that, in those earlier stages where lateral loops are most developed, the loop fragments are lacking. All the observations support the inference that loop fragments are accumulated products of lateral loop breakdown.

It is of interest in this connection that animals which had been long in captivity or on low nutritional level usually had degenerating eggs in which lateral loops have disappeared. In such eggs, loop fragments were numerous. These observations show that nuclei possess autolyzing properties

and, therefore, that attention must be paid to the feeding of the laboratory animals. The observations also show that a differential susceptibility exists between the *k*-fiber and the lateral loops. It is concluded that loop transformations in degenerating eggs are merely accentuations of normal processes that may be observed in every normal egg of the proper stage.

Nucleolar Production

As has been pointed out, the physiology of germinal vesicle chromosomes consists of two types of intricate mechanisms. The cytological evidence for the first of these, namely, lateral loop production and transformation of loops into loop fragments, has been presented in the foregoing sections. The second type, namely, nucleolar production, is discussed here.

Amphibian nucleoli are relatively large intranuclear inclusions from 6 to 25 μ in diameter. They possess multiple vacuoles that are capable of various transformations, such as fusion, eversion, and phase reversal. Under abnormal conditions, nucleoli may be made to fuse into irregular masses over 140 μ in diameter. Their general features and normal distribution are schematized in FIGURE 1. Microphotographs of them appear in all the plates, but particular attention is called to PLATES 1, 3, and 5. PLATE 1 (A and B) shows that in earlier stages they are limited to a peripheral position. Later, they take up a more central position in Stage 5 and 6 nuclei [*cf.* PLATE 1 (E)]. When the germinal vesicle breaks down in normal eggs at the time of ovulation, all the nucleoli dissolve slowly and their substance then becomes part of the cytoplasm. It would be interesting to know what role is subsequently played by these proteins. PLATE 3 (A) shows nucleoli that have migrated out into the sacs on the nuclear membrane which protrude into the cytoplasm.

All observations agreed in showing that nucleoli arise at specific loci on the chromosomes. From the point of origin they migrated to the periphery, as shown in PLATE 1 (B). As later waves of production took place between Stages 3 and 4, their numbers increased, as shown in PLATE 1 (C). Up through Stage 3, newly formed, central nucleoli were attached directly to specific chromosomes by lateral branches. Centrifugal migration appeared to be aided by expansion of the chromosome frame. It was possible to reverse the process partially by contracting the frame with dilute solutions of heavy metals, acids, and basic dyes. In cases where the nucleoli had already reached the periphery and had become adherent on the inside of the nuclear membrane, shrinkage of the frame with the major parts of the chromosomes occasionally placed severe tensions on the nucleoli via the lateral branches. Such stretched nucleoli were pear-shaped or had two cones on opposite poles. PLATE 3 (B) shows similar distortion produced by microneedles. The elasticity of the nucleolus under nearly normal conditions was readily demonstrable. When later stage nucleoli were found attached directly to the *k*-thread, as illustrated in the photograph of PLATE 5 (C), it was not possible to dissect them free without fracturing the chromosome. No by-passing of nucleoli by the *k*-thread was ever observed. It

was concluded that Stage 5 nucleoli are produced, not on branches as earlier ones were, but actually embedded in matrix substance and therefore firmly attached to the *k*-thread.

The problem of the structure and chemistry of nucleoli is very complex. The data on salt effects (*cf.* Duryee 1941) were found to conform with the principle that nucleoli are colloidal coacervates as defined by Bungenberg de Jong and Otto Bank.* It must be kept in mind that their classical observations on the morphological phenomena occurring in the case of coacervation of biocolloids are highly pertinent to the problem of nucleolar structure. For example, the typical solubility of nucleoli in dilute alkalis or in any of the common buffers can be blocked at progressive stages by appropriate neutralization. Experiments on many thousands of nucleoli have proved that nucleolar solubility is a colloidal coacervate phenomenon that takes place in stages. First, the outer shell disintegrates, freeing one or more interior droplets. Next, the inner droplets may dissolve, coalesce, or form chains, depending on the time interval between addition of the peptizing reagent and the flocculating one. The following simple rules were found that brought about internal phase reversals, eversion of contents to produce *amphinucleoli*, fragmentation, fusion, and dissolution of nucleoli.

Dissolving. Reagents like distilled water, hydroxides of Na and K (0.01 M), KCl 0.1 M, basic phosphates, bicarbonates, and the hydrating anions of the Hofmeister series (*e.g.* sulphide) all caused the nucleolar membrane to dissolve, followed by dissolution of the polyphasic interior. In all of these cases, complete disappearance of nucleoli was observed. Especially interesting was the effect of 0.1 M KCl, in which nucleoli were dissolved, but the chromosomes remained suspended in the central chromosome frame.

Fragmentation. Fragmentation of nucleoli is defined as breakdown of the nucleolar membrane with the escape or release of contained vacuoles or granules. Criteria of fragmentation could be precisely determined with an ordinary 4 mm. high-power objective. The resultant picture was a small clump of granules or vacuoles, slightly larger in diameter than the pre-existing nucleolus. Substantially, all of these reagents listed, which cause nucleolar dissolution, also produced fragmentation when added cautiously in amounts of the order of 1 $mm.^3$ per 4 $mm.^3$ of N-medium. Fragmentation instead of dissolution was also favored by slowing the rate of addition of the hydrating reagents and by arresting or neutralizing the action of the agent. NaCl 0.02 M produced smaller nucleolar droplets than did NaCl 0.05 M. Fragmentation and eventual solvation was also produced by hypertonic concentrations of the order of 0.5 M, whereas the nucleoli were always insoluble in 0.1 M NaCl. It must be concluded from these experiments that nucleoli are colloidal in character, behaving as viscous emulsoids, best described by the term "coacervates." It further follows that, since Bouin's and dilute formaldehyde also cause nucleolar fragmentation and eversion of contents, the term "amphinucleoli," used by the older cytologists, applies to a colloidal artifact.

* Bungenberg de Jong and O. Bank defined complex coacervation as "the mutual flocculation of two oppositely charged biocolloids, in which the flocculated substance has the nature of a liquid." Non-living models are formed from mixtures of negatively charged colloids such as clupeine, gelatin, serum albumin, or egg albumin, with positively charged colloids like gum arabic or Na-nucleinate.—Proc. Kong. Nederlandsche Akad. V. Wetenschappen, Vol. XLII, 1939.

Fusion. The coalescence of nucleoli was frequently observed. Several examples of fused nucleoli are shown in PLATE 1 (F). The conditions that bring about fusion were found to involve two basic factors. One is a partial or complete solvation of nucleoplasmic gel to allow the physical displacement of nucleoli. The other is surface change of the nucleolus itself that permits coalescence. These conditions are satisfied by a variety of the hydrating anions, but most efficiently by application of high dosages (60,000 r) of X rays or by NaCl solutions of the order of 0.5 M. The fact that nucleoli can coalesce is further proof of their emulsoid nature.

Eversion through the Nuclear Membrane. Experiments with dilute acids, especially H_3BO_4 0.1 M, showed that peripheral nucleoli, in contrast to the larger central ones, could be made to evert their contents through the nuclear membrane. An examination of transparent eggs (Stage 3) treated with the reagent usually showed delicate translucent hemispheres projecting 10 to 30 μ into the cytoplasm. There was always a direct one-to-one correspondence between each surface hemispherical "bubble" and each flattened nucleolus remnant on the inside of the nuclear membrane. Observations of the surface of the nuclear membrane in all the later stages revealed multiple rounded structures in the membrane substance which correspond to nucleolar remnants. These seem to warrant the conclusion that the process of nucleolar eversion through the membrane is also a mechanism by which the nuclear surface area is increased.

Discussion

The data presented in this paper, when taken together, form an integrated picture of how a chromosome functions in a cell. Lateral loop chromosomes are characteristic of the large eggs of vertebrates, notably in teleosts, amphibia, and aves. Since cleavage patterns of the egg represent primarily a distribution of materials already formed during a protracted growth period in the ovary, it is only natural to expect that the egg nucleus should possess a special mechanism for synthesizing the needed substances for later development. This is particularly important in forms, like the amphibia and fish, where the nurse cell mechanisms are not as fully developed as in the higher vertebrates. It has been brought out by this new cytological technique that there are two major methods for accumulating chromosomal products in the germinal vesicle. One method is the production from lateral loops of a large number of loop fragments, estimated to be 70,000 to 100,000. The other is by successive waves of nucleolar production, some of which result in direct contribution to the cytoplasm through the nuclear membrane, and some by belated admixture at the time of germinal vesicle breakdown. The colloidal coacervate characteristics of these nucleoli support the hypothesis that they are interaction products of oppositely charged colloids, such as nucleic acids with basic proteins. Whatever the mechanism may be, it is clear that nucleoli are produced at a limited number of loci on the chromosomes.

While the basic facts of nucleolar production described in detail by many authors are in general agreement with those reported here, there is an alternative hypothesis to explain the normal disappearance of lateral loops during

ovogenesis. This alternative can be called "retraction theory" sponsored by Ris (1945) and by Painter (1940 and 1942). It assumes, essentially, that the lateral loops are "merely gyres of the major coil of the chromonema" and that the chromosomes of the egg are not diplotene but "polytene" corresponding to a reduplicated condition proposed for the inset salivary gland chromosome. It supposes that lateral loops do not dissolve off, but are retracted back into the chromosome. Since there are at least six basic experimental and analytical reasons why the *lateral synthesis* theory developed in this paper fits the known facts better than the *retraction theory*, they are reviewed in detail.

In the first place, actual fragmentation of lateral loops into discrete filaments and granules without rupture of the chromonemata or change ln length of the chromosomes has been repeatedly observed. Among the great variety of hydrating reagents that produces this result, ordinary amphibian River solution buffered with K_2HPO_4 stands out as a physiological example. Recent experiments with X-irradiation of *Triturus pyrrhogaster* eggs at the 20,000 to 60,000 r levels gave all stages of loop fragmentation without chromonemata breaks. No case of loop retraction with any reagent has been observed. The facts of solubility and the actual observation of loop disintegration clearly disprove any possibility of retraction.

Secondly, the experiments of stretching chromosomes with microneedles did not open the lateral loops. On the contrary, multiple loop clusters were separated and could be seen to be held together by a single thread. It is recognized that the *k*-thread may have a latent cleavage plane which would correspond with tetrad formation, but indications of duplication were found only in rare instances. Conversely, it was possible to cut and tear many loops along a small segment of chromosome without affecting the stretching properties of the *k*-thread.

Third is the fact of normal decrease in number of loops with advancing ovogenesis. By late Stage 6, the average number of lateral loops per chromosome diminished toward zero, and the longest chromosome measures only 40 μ and several pair are less than 10 μ. The average thickness of each chromosome cylinder is approximately 1 μ. If the retraction theory were valid, it would be necessary to explain how the previously expanded chromosomes and loops could be compressed into such a small volume. The following example may serve to illustrate this point. A Stage 4 *Triturus* chromosome has lateral loops that project laterally an average distance of at least 15 μ. Since the loop is double, the average loop filament length is 30 μ. From the photograph in PLATE 2 (B), it was estimated that there are at least 80 loops per 100 micra of chromosome length, and the longest pair at its iso-electric point is at least 700 μ. Multiplying $30 \times 80 \times 7$ gives a total loop filament length for one chromosome at 16,800 μ or 16.8 mm. It would be impossible, obviously, for this figure to shrink to 40 μ, the length of the largest chromosome ready to go on the 1st maturation division spindle, without loss of material or extraordinary thickening. On the other hand, with the mechanism of lateral loop sloughing, as put forth in this paper, no such difficulty arises. The *k*-thread never exceeds 750 μ and it is then so

thin as to be almost unobservable under oil immersion. If such a Stage 4 chromonema is shrunk experimentally with $CaCl_2$ down to 100 μ, it does not thicken to more than 1.5 μ. On this basis alone, the retraction theory is clearly untenable. In this connection, it is of interest that Dodson (1949) has calculated the specific surface of *Amphiuma* oocyte chromosomes as 108.37 square meters per gram of tissue.

In the fourth place is the fact of variability in loop numbers per cluster. Previously, it was shown that the average number of loops per cluster diminished from 3.5 at Stage 4 to 1.7 at Stage 5, and later approached zero. It was also shown that different numbers of loops exist in the various clusters on the same chromosome. It is significant that these distribution differences along the chromosome were not noticed in the old methods of fixing, sectioning, and staining. If egg chromosomes are assumed to be diplotene, as most classical cytologists long ago agreed, then two and only two loops per cluster should appear. The fact that the loops vary from one to nine along any chromosome strongly suggests synthesis going on at different rates, rather than "gyres of the major coil." This conclusion is further borne out by the fact that lateral loops within any one cluster are usually of different sizes. When amphibian genetics and microchemical methods are more advanced, it might be expected that successive "crops" of loops might be observed and quantitatively measured.

Fifth is the problem of variation in the finer details of individual loop structure. From the standpoint of the lateral synthesis theory, granules embedded in a single loop filament represent substances produced at a specific chromsome locus and are presumably qualitatively alike. Similarly, if one assumes that there is a specific locus responsible for production of each of the complex chemicals required by the embryo—such as thyroxin, haemoglobin, and acetylcholine—then lateral loops in different clusters should contain qualitative differences. Unfortunately, microchemical methods are not yet sufficiently delicate to detect such differences. However, there is at least one, and possibly two more, morphological differences detectable between lateral loops. In *Triturus pyrrhogaster*, as has been pointed out, there is one pair of chromosomes each having a single, heavily beaded lateral loop at homologous positions. The fact that these two are markedly different from their neighbors does not fall in with the coil theory. Indeed, the point that each is a *single* structure suggests further that no question of "polyteny" is involved. The simpler explanation suffices, *i.e.*, that the heavy loop is a synthetic product of a specialized locus.

In the sixth place are the physical differences between the lateral loops and the longitudinal *k*-thread. The former are measurably more brittle, frangible, and less elastic than the chromonemata. When stretched with microneedles, any small segment of the *k*-thread could be increased from 140 to 900 per cent of its length without breaking. Lateral loop filament segments could never be stretched over 50 per cent without rupture. These facts taken together with the differential solubility data contradict the assumptions of Ris and Painter that lateral loops are portions of the chromonemata.

Finally, the *lateral synthesis* theory is adequate to explain the facts of nucleolar accumulation, production of loop fragments, and growth of the nucleus at the same time it allows for contraction of the chromosomes preparatory to going on the 1st maturation spindle. Photographic evidence has been offered showing that nucleoli originate at definite loci attached to the *k*-thread, but it is not yet known whether the matrix substance also coats the early nucleoli. It is certain, however, that nucleoli continue to increase in numbers, not haphazardly in the nucleoplasm, but in the definite area at the center of the nucleus between and immediately surrounding the chromsomes. This is also the area where loop fragments appear as the crops of loops disappear. The adoption of a chromsome formula (combinations of the letters *k*, *l*, *m*, *g*) focuses attention on the four basic units of chromsome structure: the chromonema (*k*); the self-duplicating granule or gene, as some may wish to call it (*g*); the lateral loop observed to consist of separable particles embedded in a hyaline fibril (*l*); and the chromosomal matrix (*m*) or coating material, which may also possibly contribute to the loop. The chromosome formula has already been useful in orienting microchemical experiments on nuclear components, while the retraction theory is hardly more than a sterile hypothesis based on unreliable methods.

It is relevant to this discussion that lateral chromomere loops are in some way dependent on the nutritional level of the animal as well as on the physiological state of the egg. It has often been observed that in animals which have not fed for long periods of time many of their ovarian eggs are being resorbed. Isolated nuclei from such ovaries usually exhibit ragged or fewer loops per chromosome. On the other hand, the best developed loops are seen in the appropriate stages of eggs from the healthiest individuals. Clearly, the dependence of loop structure on nutrition level supports the concept of lateral synthesis.

The experiments presented in this paper emphasize the importance of the nuclear polyphasic colloids in protecting and maintaining the chromosomes in conditions which favor lateral loop synthesis and nucleolar production. It is clear that chromosomes and nuclear substrate are highly interdependent. We are, thus, led to a general working hypothesis that the egg chromosome is a linearly expandable device to allow access of substrate to anchored template molecules or to key enzymes and thereby facilitate lateral synthesis. This view is in accord with some very recent observations on chromosome structure made by Buchholz (1947) using the electron microscope. His conclusion is that elongated longitudinal threads connect paired granules or chromomeres at the rate of 4–5/μ. From a consideration of eversion of nucleolar contents through the nuclear membrane and the widely recognized fact of normal germinal vesicle breakdown, it is apparent how chromosomal products are added to the egg cytoplasm at a very critical time preparatory to fertilization. These mechanisms might also account for accumulation in the cytoplasm of such postulated units as "cytogenes." While this paper has been confined largely to a morphological study, it has shown the fundamental dependence of nuclear morphology on the chemical constitution of the environment. Future experiments in chromosome physiology should

combine *both* colloid substrate chemistry and the experimental analysis of the units of chromosome structure. This in turn should lead to the now approachable, but still distant, goal of culturing chromosomes and their component parts outside of the cell *in vitro*.

Summary

(1) Experimental analysis of over 1600 individually isolated nuclei of amphibian ovarian eggs has shown that the chromosomes have two basic mechanisms for producing material which is later transmitted to the cytoplasm. One is the production of many thousand filaments by a process of *lateral synthesis* from multiple loci along the chromonemata. The other is by production of many hundreds of nucleoli.

(2) Techniques of isolating and washing individual nuclei and nuclear components in physiological media, followed by minimal amounts of mild reagents, have been found to yield cytological detail superior to any method of fixing and staining. Methods included microdissection, pH changes with dilute buffers, and experiments with ultraviolet light and high intensity X rays. Species used include: *Rana temporaria*, *Rana pipiens*, *Rana catesbiana*, and *Triturus pyrrhogaster* and *Triturus viridescens*.

(3) Amphibian ovocyte development is characterized by six different stages or phases of nuclear growth. A diagram of the normal series for the frog is given.

(4) The 13 pairs of anuran germinal vesicle chromosomes have constant relative size and fixed numbers of chiasmata. Each pair therefore may be recognized and identified. They are designated: Q_1, Q_2, Q_3, Q_4, R_2, R_{3a}, R_{3b}, R_4, S_4, S_6, S_7, T_1, and T_2 respectively.

(5) Experiments show the chromosomes consist of 4 basic parts: the longitudinal chromonema or *k*-thread (*k*), the lateral loops (*l*), which originate from chromonema anchored granules (*g*) and a covering matrix (*m*). Formulae for variations from the basic type (*k l m g*) are: *k m g*, *k m*, and *k g*.

(a) Lateral loops occur in separable clusters of 1 to 9 loops along a single chromonema. There is no "coiling" from one chromomere cluster to the next. Each consists of a hyaline filament with embedded granules.

(b) Lateral loops reach their greatest development in Stage 4 when the chromosome frame is most expanded. In frogs, they may extend laterally 24 μ with an average of 9.5 μ. In urodeles, comparable lateral loops average 15 μ with extremes of 2 to 36 μ.

(c) Lateral loops both normally and experimentally fragment into hyaline granules approximately 1.5 μ in diameter. Loops are not resorbed back into the chromosome. At Stage 4, loop clusters average 23 per 100 μ of chromosome length with an average of 3.5 loops per cluster. By Stage 5, there are only 1.7 loops per cluster, and, at Stage 6, the number approaches zero. The number of loops per chromosome decreases with time, although the total number of chromomeres per chromosome remains constant.

(6) Nucleoli arise at specific loci on the chromosomes. They are colloidal

in character, behaving as viscous emulsoids, best described by the term "coacervates."

(a) Nucleoli dissolve in distilled water, dilute hydroxides, and KCl 0.1 M. They are insoluble in NaCl 0.1 M.

(b) Fragmentation, with release of internal granules or vacuoles, is produced by a wide variety of hydrating anions and fixatives.

(c) Fusion of nucleoli can be caused by the action of X rays or hypertonic NaCl.

(d) Nucleoli evert their contents through the nuclear membrane, when transparent cells are treated with dilute acids. This is also a process whereby the nuclear membrane area is increased.

(7) The wide significance of this chromosome *lateral synthesis* theory in connection with the principles of germinal vesicle breakdown and preparation for fertilization is discussed. Also reviewed are the reasons for rejecting both the *loop retraction* theory and the concept of polytene strands on egg chromosomes.

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Discussion of the Paper

Dr. Hans Ris (*Johns Hopkins University, Baltimore, Md.*): One must agree with Dr. Duryee that the amphibian egg is a most interesting system and that its giant chromosomes are fascinating structures indeed. They seem to be ideal objects for the study of chromosome metabolism and nuclear-cytoplasmic interaction. Therefore, they have interested a great number of biologists since their discovery by Rückert.

The remarkable activities of the chromosomes during the growth of the egg, which Dr. Duryee just has described, are, of course, of great general interest, if they can be substantiated. However, if one has ever seen these chromosomes in the living germinal vesicle, faintly visible, tenuous strands and loops, one wonders how Dr. Duryee can be so confident in his views about their structure, based solely on observation of fresh material.

Now most cytologists will agree with Dr. Duryee that the study of living cells is of great importance and has too often been neglected; but they have also found that, for the study of the detailed structure of chromosomes, they could not rely on the observation of living material because the chromosomes are then usually very difficult to see. They have, therefore, worked out the techniques of fixing and staining chromosomes to make them more visible. I have looked at a great many different chromosomes in the fresh state and after staining and can only corroborate what Belar had already shown, namely, that fixation does not change the essential structure of chromosomes. Phase-contrast microscopy will, no doubt, extend the limits of living observation a great deal and perhaps make it possible to study chromosome structure in living cells. The cytologist will certainly want to use this wonderful new tool to confirm wherever possible in living cells what he found after fixation. But observations of living cells with the ordinary microscope alone are a very weak foundation on which to base views on chromosome structure.

In a study of these giant chromosomes in amphibian eggs after fixation and staining, I found that these chromosomes are not so totally different from other diplotene chromosomes as Dr. Duryee thinks. Like other diplotene chromosomes, they consist of several chromonemata with major and minor spirals. The gyres of the major coil form the characteristic "loops." The "loops," then, are part of the chromonemata themselves and not new structures built up by the "chromioles." Compared with most other chromosomes, the chromonemata are, however, enormously elongated. This interpretation agrees with what we know of other chromosomes. In egg cells of different insects, for instance, we can find all intermediates from small diplotene chromosomes to large ones, resembling amphibian chromo-

somes. Amphibian chromosomes are merely extreme in size, but then the salamander egg is an extreme cell also!

The dissolution and micromanipulation experiments of Dr. Duryee do not necessarely contradict this view. "Loops" may shrink or collapse upon the central core of the chromosome under the influence of the agents used and thus disappear without being dissolved. The stretching of the chromosome may break some of the chromonemata. Their "loops" would remain unstretched and they would form the observed clumps along the unbroken stretched strand.

I do not think that we can say anything at present about the way these chromosomes grow or take part in the growth of the egg and shrink again to the small size of the maturation divisions. We first need a thorough investigation of the structural changes of these chromosomes during oogenesis, making use of all cytological techniques available.

Dr. W. R. Duryee: The evidence against Dr. Ris's speculation that "the gyres of the major coil form the characteristic loops" has been presented in this paper. The fact that egg cells of insects have all intermediates from small diplotene chromosomes to large ones resembling those of amphibia is in favor of the view that lateral loop chromosomes are a widely distributed mechanism for synthesis in germ cells. One can not agree, however, with the statement that the salamander egg is an extreme cell. Compared with eggs of fish, reptiles, and birds it is both smaller and less specialized.

It is not likely that loops could disappear completely and still not be dissolved. Dr. Ris does not say how, if his theory were correct, all the "multiple" chromonemata except one breaks in every chromosome and between every cluster when the chromosome is stretched. Nor does he explain the mechanism of loop retraction. No experiment on any chromosome either within or without the nucleus has shown any retraction. All the experimental evidence is for loop fragmentation.

Dr. Ris upholds the point of view that cytologists should "confirm whereever possible in living cells what has been found after fixation." This seems like a backward philosopy. One would think, on the contrary, that the obsolete techniques of fixation and staining, if used at all, should be only to confirm what is found in the fresh, unfixed material. The experimental point of view is needed especially in the field of cytology, which has lagged so far behind its sister sciences in biology.

Plate 1 (*See opposite page*).

(A) *Rana temporaria.* Portion of ovarian wall showing young transparent eggs (Stages 1 and 2) and larger opaque eggs (Stage 3). Arrow indicates isolated nucleus from another egg (also Stage 3) which has been floated into the field.

(B) *Rana pipiens.* Young Stage 3 egg with nucleus *in situ.* Peripheral nucleoli visible beneath nuclear membrane. Microneedle inserted into center of chromosome area.

(C) *Triturus pyrrhogaster.* Stage 4 nucleus isolated in N-medium. Nucleoli embedded in chromosome frame gel.

(D) *Rana temporaria.* Stage 6 nucleus treated 0.01 M KCl to dissolve all internal structures except the chromosome frame, which coagulated with 0.005 M $CaCl_2$ appears as small ball that has sunk to the inside of the nuclear membrane.

(E) *Rana pipiens.* Early Stage 6 nucleus isolated in N-medium. Sac-like organelles protrude from the nuclear membrane. Cloud of central nucleoli surrounds and obscures the chromosome frame.

(F) *Rana temporaria.* Isolated central nucleoli and chromosome frame from a Stage 6 nucleus, coagulated with $CaCl_2$ 0.005 M.

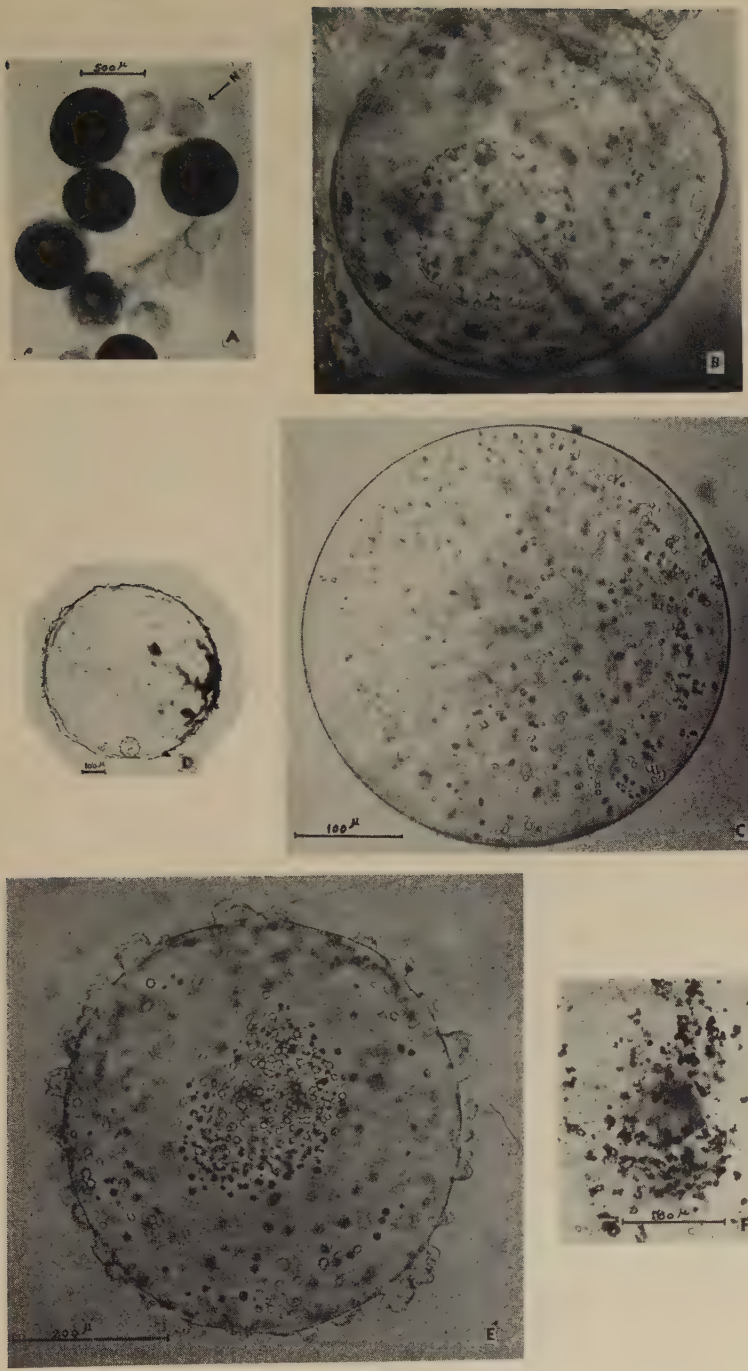


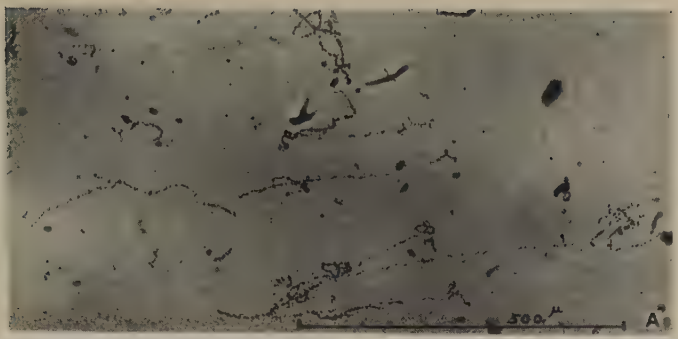
PLATE 1 *(For description see facing page).*

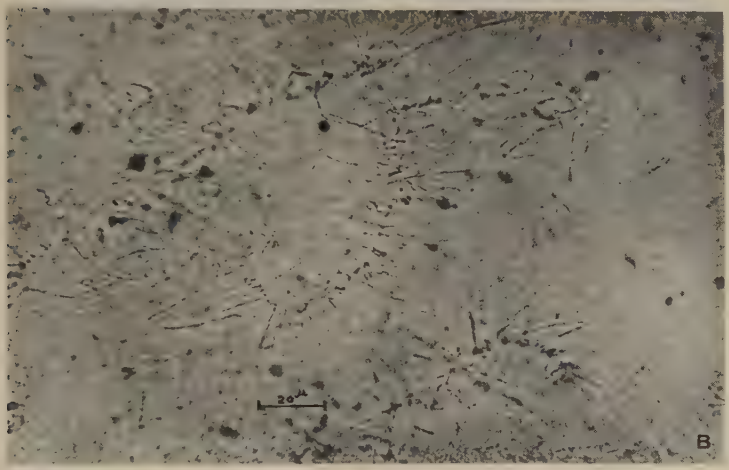
PLATE 2 (*See opposite page*).

(A) *Triturus viridescens*. Smear preparation of late stage 5 nucleus, showing long chromonemata. Nearly all lateral loops have normally previously been sloughed off.

(B) *Triturus pyrrhogaster*. Oil-immersion micro-photograph of Stage 4 chromosomes *in situ*. One portion (c. 100μ) of a chromosome pair is in sharp focus at center. Note the individual loop clusters connected by a single longitudinal thread or chromonema. Details of individual loop structure are in sharp focus at various places throughout the picture.

(C) *Triturus pyrrhogaster*. Low power view (optical section) of isolated Stage 4 nucleus. Slightly stained with 1: 8,000 crystal violet in N-medium. Note wide distribution of chromosome pairs with large lateral loops in expanded chromosome frame. Both peripheral and central nucleoli can be seen.





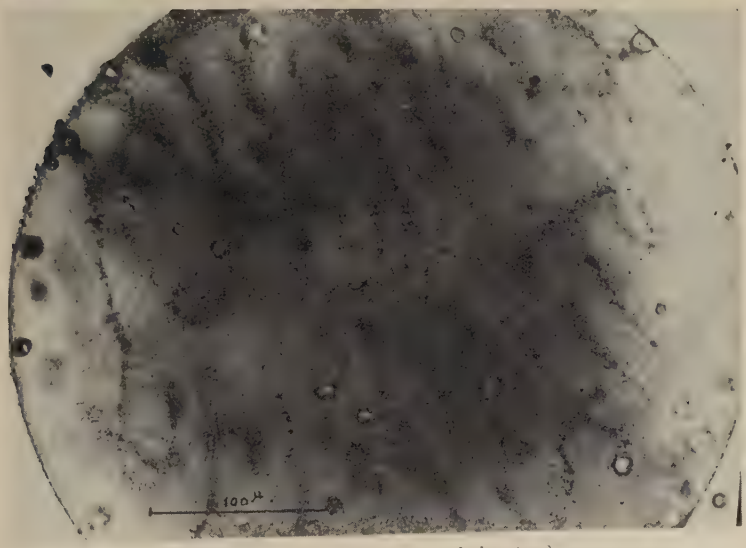


PLATE 2 (*For description see facing page*)

PLATE 3 (*See opposite page*).

(A) *Rana pipiens.* High power view of equator of Stage 6 nuclear membrane showing details of membrane sacs. A nucleolus has escaped into one sac.

(B) *Triturus pyrrhogaster.* Nucleolus being stretched with microneedles.

(C) and (D) *Triturus pyrrhogaster.* Nucleolus before and after microdissection. Note torn edge of nucleolar substance in *D* showing that at Stage 5 the emulsoid coacervate interior is a gel. The elasticity of the nucleolar capsule is also indicated.

(E) *Triturus pyrrhogaster.* Pair of Stage 5 chromosomes being stretched with microneedles. Note reduced numbers of lateral loops and their smaller relative size. Numerous nucleoli and loop fragments are visible in background

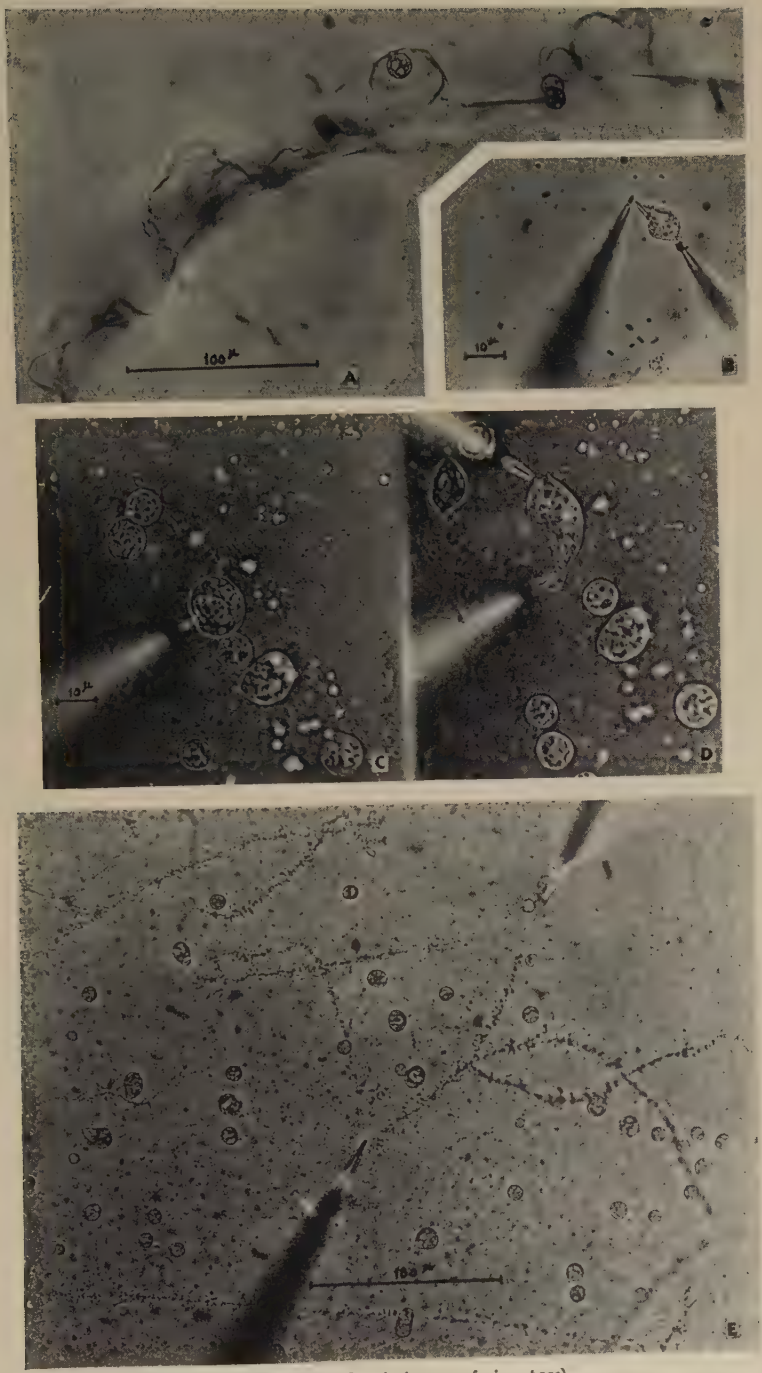


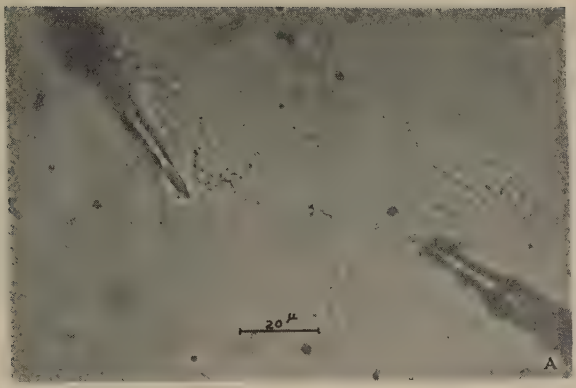
PLATE 3 (*For description see facing page*).

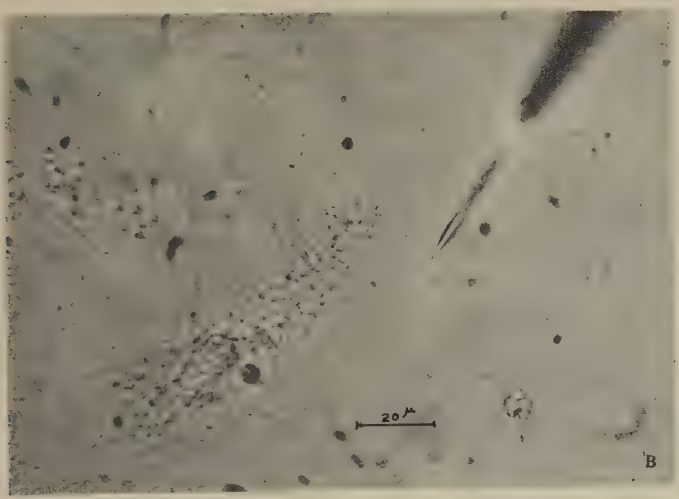
PLATE 4 (*See opposite page*).

(A) *Triturus pyrrhogaster*. Stage 4 chromosome from same nucleus shown in Plate 2 (B). Note that stretching the chromonema does not cause the lateral loops to open. Different numbers of loops can be seen in adjacent clusters. The fine chromonema appears single and altogether different from the broken loop filament projecting at right angles on the right.

(B) *Triturus pyrrhogaster*. Micro-dissection of a single lateral loop. The fine granular structure of individual loop filaments is in sharp focus at several places. Collapsed loops are matted on a small section of a chromosome now that the chromosome frame has changed to a sol. Note that the average diameter of the matted section is many times greater than that of the chromonema in (A) above. From same nucleus as (A).

(C) *Triturus pyrrhogaster*. Stage 5 chromosome being stretched with microneedles. Nearly all lateral loops have normally disappeared, but few remaining can be seen. Chromonema or k-thread is more elastic at some points than at others. If elastic limit is not exceeded, it will, on reapproximation of the needles, take on its original appearance matching the control or homologous member of the pair on the left.





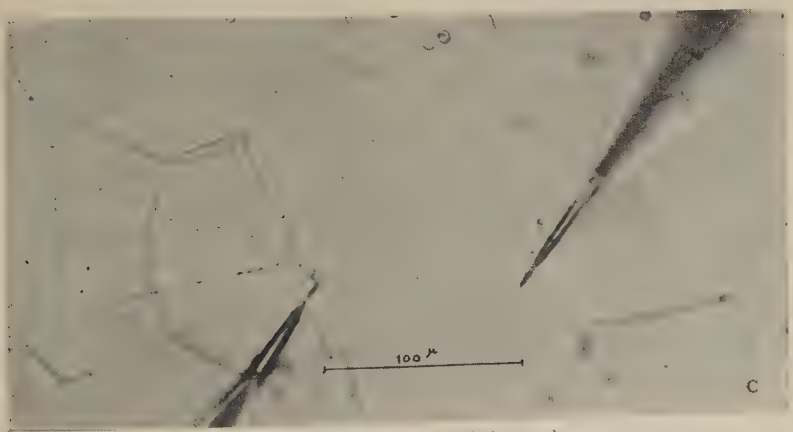


PLATE 4 (*For description see facing page*).

PLATE 5 (*See opposite page*).

(A) Cutting a Stage 6 *Triturus* chromosome. The k-thread has been severed at two places in one chromosome; the homologue is still intact. Another chromosome pair is slightly out of focus at the right. Absence of loops and presence of matrix classes this as a *k m g* type.

(B) *Triturus pyrrhogaster*. Stage 5 chromosome pair previously treated with KCl 0.01M. Loss of matrix classes this as a *k g* type.

(C) *Triturus viridescens*. Stage 4 chromosome treated with Na_3PO_4 0.1 M immediately followed by 0.003 N HCl. All lateral loops were dissolved and converted into granular fragments seen slightly out of focus in background. Note the large terminal nucleolus. (Oil-immersion.)

(D) *Triturus pyrrhogaster*. Ring or "Q" type chromosome pair from Stage 6 nucleus. Note swollen matrix, granules and the k-thread. Also the absence of loops. *k m g* type.

(E) *Triturus pyrrhogaster*. Stage 5 chromosome pair which had many lateral loops before receiving 100,000 r of X-rays. Note absence of lateral loops but persistence of the k-thread. Also the high degree of correspondence between granules in each chromosome.

(F) *Rana pipiens*. Transformation of a Stage 4 *k l m g* type into type *k g*. Isolated chromosome pair treated with Na_3PO_4 0.1 M then acidified with HCl. Note loop fragments in background and correspondence of granules in the k-thread at right of chiasma.

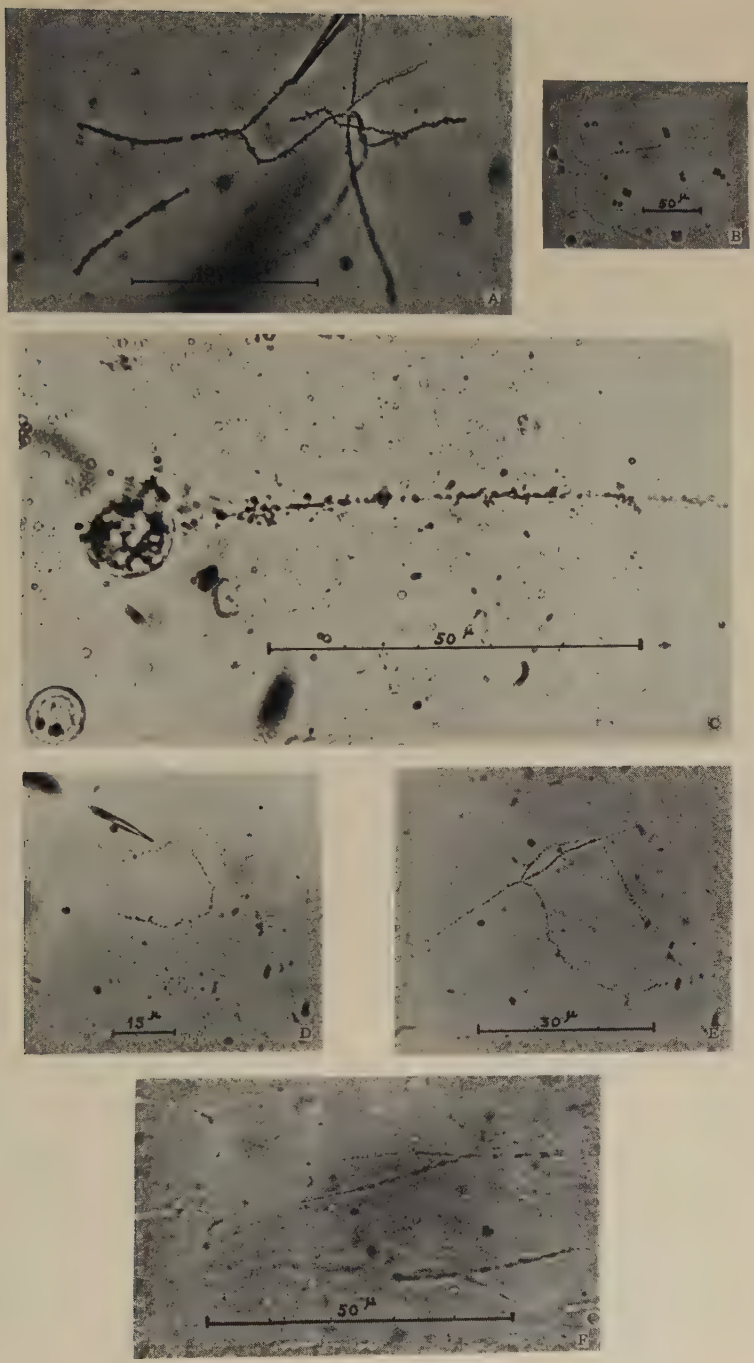
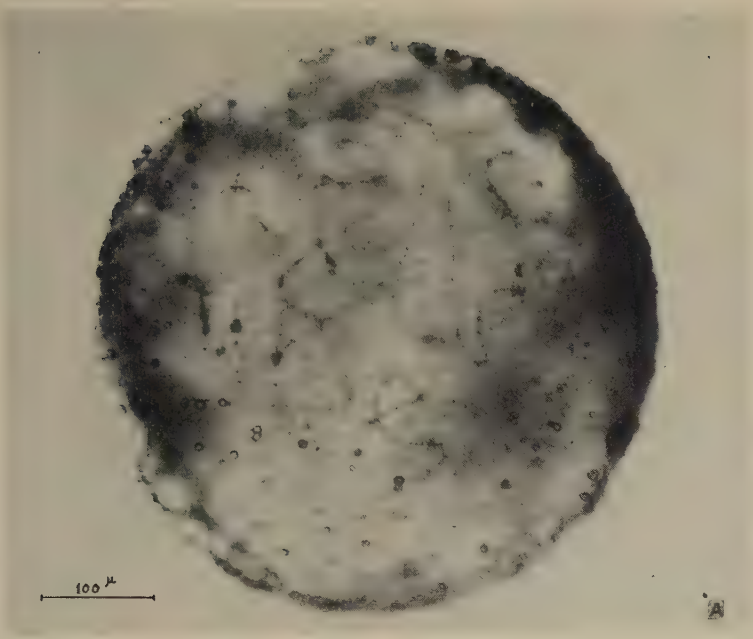


PLATE 5 (*For description see facing page*).

PLATE 6 (*See opposite page*).

(A) *Rana catesbiana*. Stage 5 nucleus isolated in N-medium and brought to iso-electric point of pH 4.5. Chromosome pairs have only a few lateral loops at this stage. Accumulation of loop fragments adjacent to chromosomes is very marked. Note peripheral nucleoli in area outside of the chromosome frame.

(B) *Rana catesbiana*. High power view of central area of Stage 5 nucleus. Large numbers of loop fragments are visible between the chromosomes, which are now of the *k m g* type. Nucleoli are shown immediately outside the central chromosome frame.



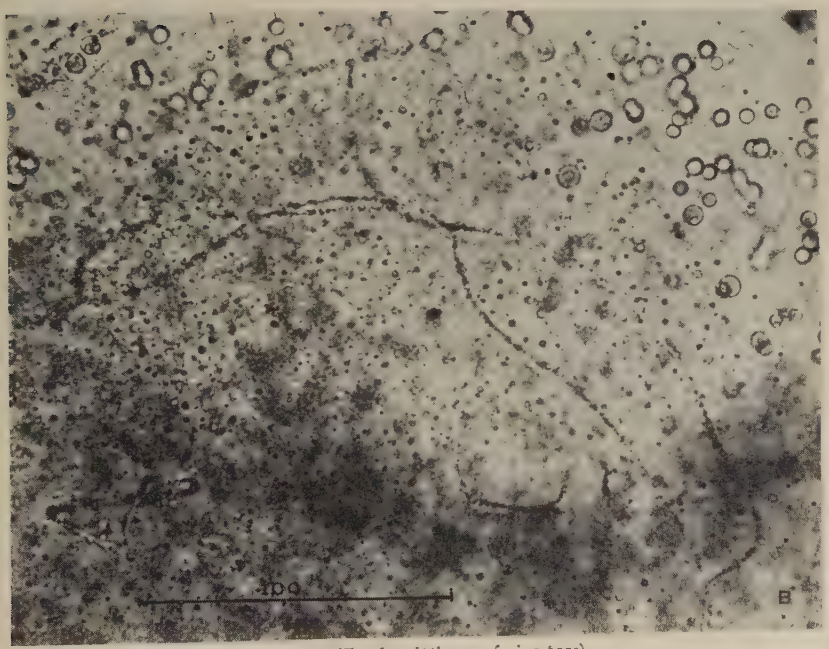


PLATE 6 (*For description see facing page*).

FIBER PROTEIN STRUCTURE IN CHROMOSOMES AND RELATED INVESTIGATIONS ON PROTEIN FIBERS

By Daniel Mazia*
University of Missouri, Columbia, Missouri

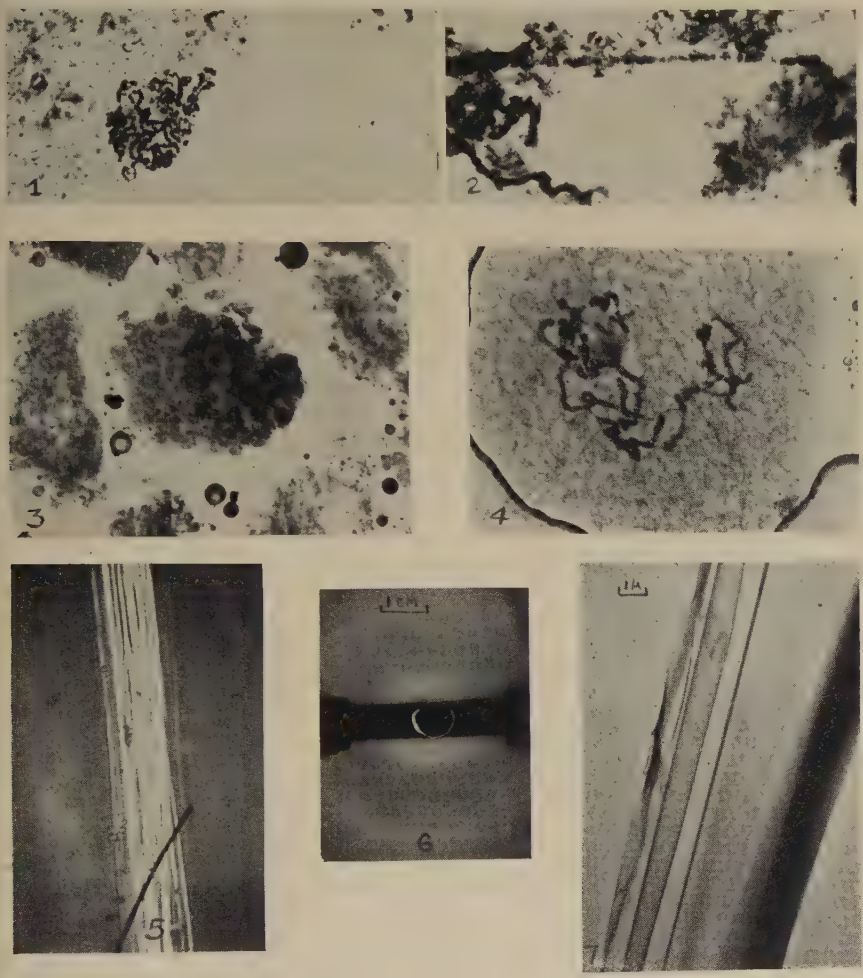
The numerous problems of chromosome function fall into two major groups: (a) those concerning the chemical composition of the chromosome and directed toward an ultimate comprehension of the nature of genic material and (b) those concerning the molecular architecture of the chromosomes as expressed in their visible and mechanical properties, their movements and reproduction in mitosis and meiosis, and the phenomena of breakage and recombination. This paper is concerned with the latter question at its most elementary level, the question of the intermolecular forces responsible for the organization of proteins and nucleoproteins into the tangible, elastic, "insoluble" body observed under the microscope, and subject to manipulation.[1]

Direct means are not available for a study of this question, nor, because the classes of compounds found in chromosomes are generally studied in solutions, do we have even a clear conception of the forces involved in the realization of structure at this level.

The action of proteolytic enzymes on dipteran salivary gland chromosomes has been studied from this point of view.[2] Once it was shown that the nucleic acids, as such, did not seem to be essential to the maintenance of the form and material continuity of salivary gland chromosomes, it was possible, applying knowledge of the peptide specificity of certain proteases,[3] to consider the protein constitution of the chromosomes in relation to their structure. The basic observation is that of Caspersson[4] that trypsin effects complete dissolution of the chromosomes, implying a continuous structure of proteins containing basic amino acids. The action of crystalline pepsin, shown in FIGURES 1 and 2, presents a different picture. Represented in FIGURE 1 is the action of crystalline pepsin (5 mg. per ml. at pH 1.5 for 15 minutes) on chromosomes in the whole freshly dissected gland of *Drosophila melanogaster*. The control, FIGURE 2, was exposed to HCl of pH 1.5 alone for the same time. The effect of enzyme is to cause a drastic shrinkage of the chromosomes to less than half of their original volume. The loss of material is largely in the interband regions, these virtually disappearing as the bands pull together. Some of the digested material is also contained in the bands, which become narrower. The general impression is that the enzyme has not affected the staining desoxyribonucleoprotein of the chromosomes, but has removed another component which is normally evident in the interband regions, a protein which contains little or no nucleic acid, but which occupies the larger part of the chromosome volume. It is to be noted that the removal of this large volume of material does not cause any breaks

* The author wishes to acknowledge the assistance of the American Cancer Society, through the Committee on Growth of the National Research Council, which made much of this work possible. The unpublished portions of the investigations summarized here will be published in collaboration with Dr. Teru Hayashi, now of Columbia University, and Mr. Kenneth Yudowith, Department of Physics, University of Missouri.

in the continuity of the chromosome. It remains a miniature version of the original structure, in fact, approaching "normal" chromosomes in size. The



FIGURES 1–7

FIGURE 1. Chromosomes from gland digested with pepsin (5 mg. crystalline pepsin per ml., pH 1.5, 25°C, 15 minutes) Acetocarmine.

FIGURE 2. Pepsin control. Gland treated with HCl alone for 15 minutes. Time, temperature, and pH same as in FIGURE 1.

FIGURE 3. Control to cathepsin experiment. Gland immersed for 30 hours in buffer pH 3.5 containing 1 mg. per ml. of NaCN.

FIGURE 4. Chromosomes from gland treated with cathepsin solution. (Activity described in text.) 30 hours at 35°C.

FIGURE 5. Polarized light view of surface-compressed albumin fiber.

FIGURE 6. Wide-angle X-ray diffraction of albumin fibers. Ni-filtered Cu radiation (wavelength 1.54 Å) Camera radius 5.377 cm.

FIGURE 7. Electron microscope view of portion of albumin fiber not cast.

experiment is easily interpreted on the basis of the peptide specificity of the enzyme. Pepsin is specific for peptide linkages involving an acidic amino

acid residue and is inhibited by the proximity of a basic group. It would not be expected, therefore, to digest many linkages in a protein of the histone type, but to affect proteins with neutral or acidic isoelectric points. It is evident, therefore, that the salivary gland chromosomes possess a dual composition, a continuous structure of histone-like protein carrying the nucleic acid running through a continuous body of relatively acidic proteins containing insufficient nucleic acid to stain appreciably with basic stains. The nucleoprotein portion is obviously concentrated in the bands and runs through the interband region in the form of strands. The acid protein is more concentrated in the interband regions, though accounting for a considerable part of the band volume as well. The nucleoprotein represents a continuous architectural framework, since removal of the other component does not interrupt the typical structure. Whether the acid-protein constituent has a parallel structural significance cannot be determined enzymatically, since trypsin, which digests nucleohistone, also affects most acid proteins.

Extending this enzymatic "dissection," attempts were made to digest the salivary chromosomes with various intracellular proteases in the crude form of tissue autolysates. The crude extracts proving remarkably ineffective, a purified cathepsin[5] preparation was tested, and again, in spite of variations of time, concentration, pH, and activators, the chromosome structure appeared unaltered. FIGURE 3 shows a chromosome from control gland, treated for 24 hours at 35°C with buffer of pH 3.5 containing activator but no cathepsin. FIGURE 5 shows chromosomes treated at the same temperature for the same time with cathepsin solution (52×10^{-4} Hb units per ml.[5]). Not only are the chromosomes not digested, but the enzyme-treated glands make a superior preparation from the cytological standpoint, because the enzyme has apparently digested nuclear and cytoplasmic proteins that normally interfere with the spreading of the chromosomes after such long treatments. Since the experiments with trypsin and pepsin, as well as chemical extractions[6] and ultraviolet absorption data[7] have characterized the classes of protein present in the chromosomes, their digestibility when in solution may be measured quantitatively. The activity of our cathepsin preparation on hemoglobin (as an example of a pepsin-digestible protein) was determined by the standard method.[5] An activity of 52×10^{-4} Hb units per ml. was obtained at 35°. The activity was also tested on a solution of nucleohistone (from thymus) in 1M NaCl, the measure of activity being the amount of nucleic acid rendered soluble in water at pH 6.0. Ninety per cent of the nucleic acid became soluble within 24 hours at 35°C. Since the substrate remained very viscous throughout, the effect of the enzyme was evidently proteolytic, rather than a depolymerization of the nucleic acid. It should be noted that this digestion by cathepsin of both acidic and basic proteins is to be anticipated from Bermann and Fruton's specificity data.[3]

If cathepsin is capable of digesting solutions of the types of proteins which, upon reasonably good evidence, seem to constitute the major portion of the salivary gland chromosome, the problem is to explain why the chromosomes themselves are unaffected. One possibility would be that the chromo-

some proteins are insulated in some way by layers of lipid or nucleic acid,[8] but this seems to be excluded by the positive results obtained with pepsin and trypsin. Another possibility is that the factor of structural organization as well as peptide composition is determining the reaction of the proteins to the enzyme. There is a precedent for this in the observation of Goddard and Michaelis[9] that keratin, which is not digested by trypsin in its original fibrous form, becomes digestible when put into solution by treatment with thioglycolic acid. The physical organization, not the reduction of the S, is the determining factor, for when the S is reoxidized, the protein remains soluble and also remains digestible by trypsin.

The possibility that the chromosomal proteins are in the fibrous state is suggested by every obvious property. Chromosomes are not easily soluble, are elongate and elastic,[1] and resolve microscopically into distinct threads. The term fibrous no longer implies a distinct class of proteins, but merely a type of physical organization, since it has been shown repeatedly that corpuscular proteins may be converted into fibers. In fact a branch of the textile industry has been built around this transformation. Therefore, it is possible to determine whether the digestibility of almost any protein differs when it is in the soluble, corpuscular state and in the fibrous state. Of the methods available for conversion of corpuscular to fibrous proteins, one that seemed feasible under conditions in the cell was chosen: the unfolding of the molecule to form a monolayer of "surface-denatured" protein and the compression of the film into a distinct fiber, as originally described by Devaux. The procedure is analogous to what Kopac, in this monograph, has called the "Devaux effect," except that in our procedure the film was compressed in one dimension by barriers. Fibers of a variety of proteins have been prepared by this method: ovalbumin, histone, hemoglobin, nucleohistone, casein, *etc.* The digestibility of the fibers, as evidenced by visible disintegration, was compared with the digestion as determined by chemical methods. Their behavior toward trypsin and pepsin followed the expectations from their peptide composition. Trypsin digested all the fibers tested. Pepsin digested fibers of hemoglobin and more acid proteins, but had no observable effect on histone or nucleohistone fibers. It could even be shown that when a fiber composed of a mixture of nucleohistone and albumin was treated with pepsin, only the albumin was digested and the fiber underwent shrinkage. With cathepsin, the artificial fibers, just as did the chromosomes, behaved in an anomalous fashion. None of the fibers was digested, even though the protein solutions from which the fibers were prepared were attacked. Therefore, making allowances for the fact that we are dealing with an impure enzyme preparation, the most obvious conclusion is that some structural change involved in the transformation of proteins into the fibrous state renders them resistant to hydrolysis by cathepsin. In so far as conclusions can be drawn from negative results, the parallel behavior of chromosomes and artificial fibers of types of proteins composing chromosomes is taken to indicate the possibility that their basic structural organization is comparable. Further refinement may possibly make digestibility by cathepsin a test for proteins in the fibrous state. At the very least, this

evidence adds a somewhat more direct argument to the inference already drawn by other workers, summarized by Bateman,[10] that the physical properties of chromosomes derive from the fibrous state of proteins.

Most of our knowledge of the structure of the protein fibers is based on work with natural fibers of relatively simple and biologically inactive proteins. Assuming the possible validity of the parallelism between the molecular structure of chromosomes and of protein and nucleoprotein fibers prepared by the technique described, the structure and biological reactivity of these fibers has been investigated. The results, as will be seen, give some insight into the biological possibilities of the fibrous state of proteins independently of the application to the special case of chromosomes.

Microscopically, the surface-compressed fibers appear as bundles of fine, refractile fibrils, even under the highest magnifications of the light microscope. They are highly birefringent, as shown in FIGURE 5, which represents a portion of an ovalbumin fiber. Measurements of birefringence of wet fibers yield a value of 0.002, positive with respect to the fiber axis. This is rather a high value when allowance is made for the water content, and it must be concluded that some element in the structure is oriented. The material lends itself very well to electron microscope observation. FIGURE 7 is a view of a portion of a fiber of ovalbumin. The fibers of other proteins, including nucleoproteins, were essentially similar in appearance. The fibrillar appearance of the fibers under the light microscope turns out to be an illusion resulting from folding. The fibers, which were prepared by compressing, monomolecular sheets, remain sheets. The striations are the product of multiple thicknesses of the unit sheet, which is very thin. Whether it is actually monomolecular cannot be ascertained, since there is no means of measurement of thickness. Nothing in the observations excludes the possibility.

Since the folded sheets do show positive birefringence, their X-ray diffraction was investigated, with a view to determining the type of transformation effected by the unfolding at the surface. Facilities only for wide-angle diffraction were available, but these were expected to be adequate, since something analogous to Astbury's keratin patterns was anticipated. The actual results of an exposure of a bundle of parallel ovalbumin fibers (wavelength 1.54 A, camera radius 5.377 cm.) appear in FIGURE 6. The rings correspond to spacings of 3.2 and 4.2 Angstroms, with a suggestion of a spacing of 10–12 A, *but there is no evidence of orientation.* These roughly correspond to the beta keratin spacings which have also been observed in denatured albumin. The best interpretation that seems possible from these data is that the fibrous structure does not depend upon a grid of polypeptide chains parallel to the fiber axis. The evidence of the spacings described would indicate that the chains are at least in part extended, and the extended chains folded in a two-dimensional pattern that has no compulsory relation to the fiber axis and, therefore, is potentially specific. It is easiest to imagine the fiber as a grid of flattened molecules, the orientation of whole molecules being responsible for the orientation observed with polarized light. If this is the case, evidence of orientation with large spacings should be ex-

pected in small-angle diffraction measurements. Such measurements have been undertaken.

With respect to the general problem of fiber structure, probably the main significance of these observations derives from the fact that proteins may form strong, elastic stable fibers by linkage of two-dimensionalized individual molecules without the drastic arrangements that would be required by the keratin picture. It is of further interest that the molecular units within the fiber can be low-molecular weight proteins and that the fibers may have a mixed composition. There need not, therefore, be any chemical difference, but only a difference in physical organization, between "structure" proteins (a term usually limited to highly asymmetric large molecules) and the soluble proteins of the cell. With respect to the immediate problem of chromosome structure, it is evident that the type of fiber structure visualized corresponds closely to the requirements of the simplest hypothesis of chromosome duplication, namely, that the original molecules provide the pattern upon which the daughter molecules are synthesized. This cannot easily be conceived except in terms of an essentially two-dimensional molecular structure. The picture of the chromosome thread as a microscopically and mechanically fibrous structure that is, submicroscopically, a folded film meets these requirements. The fact that this is true, of course, does not constitute proof of the hypothesis, but the hypothesis does establish the physical plausibility of concepts of chromosome duplications that have seemed, to some, to be required by the facts of genetics and cytology.

Since the proposed structure depends on establishment of a fibrous condition by "surface denaturation" (and any interface between an aqueous and non-aqueous phase is adequate), the difficulty presents itself at once that such a structure might be incompatible with the specific reactions, which are believed to take place in the chromosome, dependent on the "native" state of the enzymes involved. Even assuming that "surface denaturation" does not necessarily lead to loss of specificity, an assumption for which some evidence has been available,[11] interaction of constituents of these rather solid structures might present problems not ordinarily visualized in the kinetic studies of enzyme action in solution. Yet, such interaction presumably takes place in the chromosome, expressed as "position effects" and similar phenomena. It has proved to be relatively simple to investigate the possibility and the properties of enzyme-substrate reactions when both are contained in a solid fibrous structure.

By using a mixture of proteins as starting material, one may prepare films and fibers containing the proteins in approximately their original proportions. The composition of the film may be verified by determining with the surface balance the area occupied by a given amount of mixed protein and comparing this with the expected area obtained by separate spreading of the components of the mixture. In the present case, mixtures of crystalline pepsin and crystalline ovalbumin were spread over buffer of pH 4.2, where the enzyme is inactive and spreading is satisfactory. The basic facts that the enzyme has not lost its activity and that it may react with the substrate when both are contained in the solid phase of a fiber are demonstrated visu-

ally by placing the complex fiber in a medium of pH 1.5. Under the microscope, the fiber is observed to digest itself and to disappear in a short time. For example, a fiber with an albumin-pepsin ratio of 20 to 1 disappears in about one minute at pH 1.5. This microscopic method of estimating enzyme activity from the autolysis time becomes fairly reproducible, and it is possible to obtain a pH optimum curve, a measure of the relation between proportions of enzyme and substrate, to observe that the system is susceptible to heat denaturation, *etc.*

However, for assurance that this visible disintegration of the fibers actually is the result of hydrolysis of peptide linkages, it is necessary to employ a chemical method. Pepsin albumin fibers were collected in quantity,

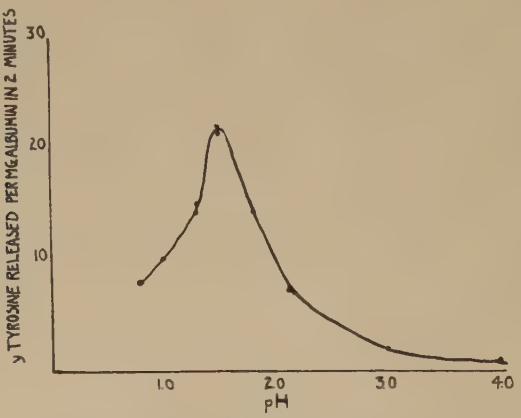


FIGURE 8. The pH dependence of the digestion of an albumin-pepsin film. Enzyme-substrate ratio 20:1. Temperature 30°C. Ordinates: gamma "tyrosine color equivalents" produced per mg. of substrate in two minutes. (Tyrosine color equivalent is the amount of digestion products giving color with the Folin-Ciocalteau reagent, expressed in terms of the amount of tyrosine given the same color value.)

and weighed out in 1 mg. samples, which were exposed to 0.3 ml. of the desired buffer for the experimental period. The reaction was stopped by trichloracetic acid, and filtration and colorimetric determinations of split products yielding color with the Folin-Ciocalteau reagent followed on a micro scale the Anson method.[12]

There is no doubt that the pepsin digests peptide linkages in the albumin when both are contained in a surface-compressed fiber. FIGURE 8 indicates both that peptides soluble in trichloracetic are produced and that the pepsin action has about the same pH optimum as when acting in solution.

The rate of digestion as the proportion of substrate to enzyme is varied is shown in FIGURE 9. It is evident that the initial rate of action is high. This is the part of the reaction that presumably takes place in the solid fiber. The later slow reaction probably reflects the action of the pepsin liberated from the fiber on the fragments remaining after visible disintegration. It is apparent, also, that digestion takes place even when the ratio of substrate

to enzyme is as high as 80–1. From the standpoint of the problem of enzyme reactions in solid phase systems—a question of importance to the cell physiologist independent of the applicability of this particular system to the special case of chromosomes—these data raise some interesting questions. The geometry of the system is such that the unfolded molecules would seem to be in contact with each other only at their margins. Apparently, an enzyme can activate a substrate molecule in this relationship. It does not seem likely that an enzyme molecule in the film can be in contact with as many as 40 substrate molecules. It would be anticipated that the first part of the reaction would, at most, involve the action of the enzyme on its few neighboring substrate molecules, the enzyme then being liberated into the medium leaving a continuous fiber with holes in it. Actually, the fiber

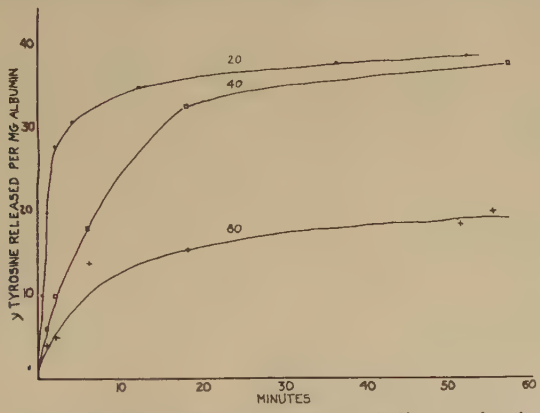


FIGURE 9. Rate of digestion of albumin-pepsin fibers as a function of substrate-enzyme ratio (numbers over curves). Ratios represent absolute amounts of crystalline albumin and crystalline pepsin. Ordinates: tyrosine color equivalents of split-products soluble in 6 per cent trichloroacetic acid.

disintegrates early in the reaction and the analytical data indicate that a large proportion of the ultimate products is released in the early rapid phase. It cannot be postulated that the liberated enzyme is, after digesting its way out of the film, acting from the bulk medium, because, as will be seen in FIGURE 10, the action of dissolved pepsin is much slower than that observed in the complex fibers. One possible explanation is that the liberated pepsin is trapped in the layers of the fiber mass and, therefore, acts from small volumes of high "concentration." This is to be tested by observing the reaction in very thin films, employing the electron microscope. Another possible explanation is that the pepsin is not liberated into the liquid phase, but works its way around an ever-widening hole in the film, always attached at some points. Finally, the work of Rothen[13] renders plausible the most obvious explanation of these results: that the enzyme need not be in contact with substrate molecule. In fact, the pepsin-albumin fiber system, composed as it is of closely linked protein molecules, would seem to make less demands on the "energy continuum" concept of long-range action than does

the Rothen experiment on the action of trypsin through layers of Formvar. The crudity of these concepts and the fact that one finds it impossible to apply to this system the very term "concentration," and yet has no substitute, merely illustrate how badly cell physiologists, who have to deal with the gross as well as the microheterogeneity of the cell, require the development of a chemistry of reactions in structurally restricted systems.

FIGURE 10 shows how the structure of the pepsin-albumin system may affect the rate of reaction. In these experiments, the amount of substrate was the same (1.0 mg.) and the total volume of reaction mixture was the same (0.3 ml.). The amount of enzyme was greater in the case where pepsin in solution was acting on fibrous albumin. It is evident that the reaction proceeds much faster when both enzyme and substrate are contained in the fiber.

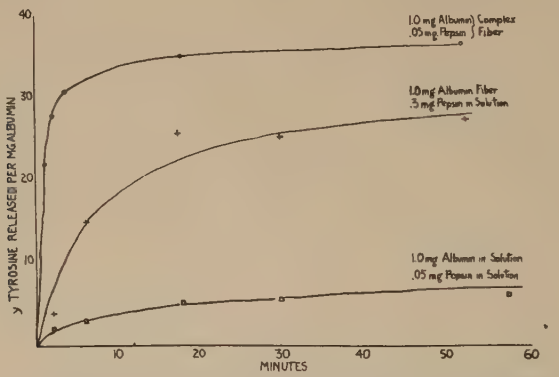


FIGURE 10. Rate of digestion of albumin-pepsin fibers compared with digestion of albumin fibers by dissolved pepsin and dissolved heat-denatured albumin by dissolved pepsin. Ordinates: tyrosine color equivalents of split products soluble in 6 per cent trichloroacetic acid.

Thus, far from causing loss of activity, transformation of the enzyme-substrate system from the soluble to the fibrous state actually increases the rate of activity. One might predict that this would be the case because the reactants are "concentrated" in the fiber. One might also predict the reverse result, not only because of the factor of surface denaturation, but because of the limited mobilities in the solid phase. In any event, the rate of activity would not be predictable from information as to the amount of enzyme and substrate and the volume of the system. The configurational factor would have to be known. These observations permit us to investigate the possibilities of a fibrous but, essentially, two-dimensional molecular structure for chromosomes, with the assurance that enzyme reactions can take place within such a system and at a high rate.

Since the earlier work on the activity of surface denatured proteins, the question has always been raised whether the specific activity found may be attributed to adsorbed unspread protein or partially spread protein in the film. As to the latter, it is now the opinion of some surface chemists[14] that the spreading is an all-or-none phenomenon, the varying "thicknesses" re-

ported in earlier papers representing merely varying proportions of material going into the film. As for adsorbed pepsin, we were unable to find evidence of it in experiments where pepsin solution was injected under an albumin film. After the thorough washing to which all our fibers were subjected, the albumin fibers remained inactive. The discussion of this question is rather involved, and will be presented in another place.[15] Here, it need only be said that we have found no evidence contrary to the conclusion that in the complex films and fibers the pepsin molecules are unfolded, and much evidence in favor of it.

Of the distinctive properties of chromosomes which seem to derive from their structural organization rather than from their composition, perhaps the most workable is their sensitivity to radiations. X-ray doses of a few to a few hundred r units cause chromosome breakage and mutations, while *in vitro* experiments on proteins and nucleic acids show that tens or hundreds of thousands of r units are required to effect a measurable chemical change. The experiments of Dale,[16] showing effects of small doses, presuppose a dilute and pure solution of enzyme protein, a condition that would hardly be expected in the cell. It seemed possible that the sensitivity of chromosomes might be due to the susceptibility of intermolecular linkages involved in their mechanical structure rather than intramolecular configuration. To test this possibility on a simple system, we have irradiated albumin and pepsin-albumin films and determined their mechanical properties as expressed by force-area curves. The results were negative up to 6000 r. The compressibility of the film remained unaltered. The next possibility was that radiation might affect the enzyme-substrate linkage or, returning to the original concept, the enzyme itself. It is a very simple matter to irradiate a pepsin-albumin film, compress it into a fiber, and estimate its activity in terms of the time required for visible disintegration. These estimations become quite reproducible with experience, although obviously they must be checked by the quantitative method.

The results obtained in preliminary experiments do indicate a close correlation between the structural configuration and the sensitivity to radiation. In the control experiments, the 20:1 albumin pepsin solution (pH 4.2, total protein 5 mg. per ml.) was given as much as 6000 r (Al filtered Cu radiation) and then spread on buffer of pH 4.2. The film was compressed into a fiber and the digestion determined by applying HCl of pH 1.5. The digestion time of unirradiated material ranged from 45 seconds to 70 seconds. The digestion time of the irradiated material fell within the same range. These results are not surprising, since very much higher doses are generally required to cause enzyme inactivation in relatively concentrated solutions and in the presence of a large amount of other protein. When the same mixture is first spread, then irradiated, and finally compressed and tested for activity, the effectiveness of the radiation is very different. After a dose of 390 r (the dosage data are approximate, based on a previous calibration of this tube), the fibers no longer digested at all in HCl of pH 1.5. After a dose of 140 r, the digestion time was 10 minutes as compared with a control (unirradiated) of 45 to 70 seconds. After 70 r, the digestion time was 4–5

minutes. It is evident that even with this crude method of measurement, the effects of a dose as small as 70 r were evident outside the possibility of error.

Several interpretations of the greatly increased sensitivity resulting from spreading the protein are possible. One is that the pepsin-albumin intermolecular linkages are altered and cannot reform (so far, these effects have all appeared to be irreversible). A second possibility is that, after surface denaturation, a relatively small amount of energy is required to complete denaturation to the point of loss of specificity, a possibility that we are testing in a study of heat denaturation. A third possibility centers around the increased exposure of the unfolded molecules to irradiation products in the medium. This system may offer an interesting tool for the study of irradiation effects on proteins. Obviously, the results conform to the possibility suggested by the earlier part of the investigation, that of the essentially sheet-like structure of the chromosome fiber.

Summarizing, this paper is not presented as a hypothesis of chromosome structure. Such a hypothesis would be useful in cytological and genetic thinking only when it was based on more direct work on the molecular structure of chromosomes themselves. Lacking means for such direct observation, we can only explore those possibilities which conform to existing cytogenetic and biochemical information and which may reveal those properties of chromosomal constituents which emerge when those constituents are organized in a form that at least parallels the gross mechanical properties of the chromosomes.

Summary

(1) The action of trypsin, pepsin, and cathepsin on salivary gland chromosomes is described. The chromosomes are not digestible by a cathepsin preparation which is quite active against dissolved protein and nucleoprotein substrates.

(2) The non-digestibility by cathepsin seems to depend on the fibrous organization of the chromosomal proteins. Proteins and nucleoproteins which are digested in solution become indigestible after conversion to the fibrous condition by compression of monolayers.

(3) The structure of surface-compressed protein fibers has been investigated. The birefringent fibers, which appear to be composed of fine fibrils, on electron microscopic examination, turn out to be highly folded thin sheets, possibly monomolecular. The fibers, in X-ray diffraction measurements, give the spacings of the beta keratin system but no pattern indicating orientation. It is suggested that the fibers are composed of chains or grids of linked molecules, a pattern of polypeptide chain folding within the molecule being preserved.

(4) Fibers composed of pepsin and albumin are highly active enzymatically. The activity is higher than that for the same amounts of enzyme and substrate contained in solution in the same volume.

(5) The complex films of pepsin and albumin may be inactivated by small doses of X-radiation which have no effect on comparable pepsin-albumin solutions.

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Discussion of the Paper

Dr. Kurt G. Stern (*Polytechnic Institute of Brooklyn, Brooklyn, N. Y.*): Interesting and ingenious as the experiments here reported by Dr. Mazia are, it is difficult to see how they could throw light on the fine structure of chromosomes. Even fibers prepared from desoxyribonucleoprotein would tend to oversimplify the picture which the cytologists have developed for mitotic chromosomes. Dr. Mazia's experiments suffer from the same objection as all other model experiments of this general type: they tend to explain little and to lure those interested in the actual biological structures into drawing untenable analogies. A case in point are the recent model experiments where "nerve action potentials" were elicited by adding acetylcholine to model systems composed of oil-water interfaces.

Dr. D. Mazia: As a biologist who has been quite actively interested in "the actual biological structures," I should like to emphasize that there was no intention here of presenting a model of a chromosome in the sense of a working imitation. I agree with Dr. Stern on the essential sterility of such an approach. But, just as he has so fruitfully studied *in vitro* the properties of nucleoproteins extracted from nuclei, hoping to gain insight into the nature of gene material, we have examined the structural implications of both chromosomal nucleoproteins and other proteins hoping to learn something about how these materials may be organized at the supramolecular level into the tangible chromosomes we have been studying for a long time. We have done quite a bit of work on desoxyribonucleoproteins (see Ref. 15) and find that the generalizations made in this paper with regard to protein fiber structure apply to them.

We have dealt with non-nucleoproteins as well, because, as pointed out in this paper, they comprise a considerable part of the chromosome material and, as Mirsky has shown recently, have an important part in chromosome structure. This is especially evident in our experiments with pepsin. For the study of activity of proteins in fibrous bodies, we have to use non-nucleo-

proteins, since we have no nucleoprotein, with the possible exception of Avery's "transforming principle," whose activity can be measured. As for misleading biologists, the ideas presented here—and I think anyone familiar with the literature of genetics will agree with me—have been part of the theoretical thinking of geneticists for a long time. Our contribution has been only to demonstrate, if we are correct, that these ideas, particularly that of the two-dimensional configuration of self-duplicating units, are chemically possible. In brief, we are interested in the problem of how the materials which Dr. Stern and others study in the test-tube can become chromosomes in the cells. Lacking direct means of approach, we have explored the possibilities of fibrous organization and tested these by parallel observations on artificial fibers and on chromosomes. If one must use the term "model" at all, our fibers have been used as models of a *kind* of protein organization whose properties may be applicable to the chromosome. This is quite different from a model of a chromosome itself.

Dr. Anna Goldfeder (*New York University, New York, N. Y.*): The question arose as to under what conditions the X-radiations were applied to the pepsin albumin fibers and to the various substrates.

In my tissue culture studies, I found that when tissue fragments were left in a nutritive medium of chick embryo extract, chick plasma, and tyrode solution, a lesser dose of X-radiation was required to prevent proliferation than when the fragments were removed from the medium immediately after radiation, washed in tyrode solution, and transferred into a fresh medium. Moreover, when the components of the nutritive medium were irradiated with the same dose separately and later used for cultivation, no apparent effect on the growth of the tissue was observed. Consequently, it might be concluded that some toxic substances are produced during radiation which can be removed by washing the irradiated tissue fragments in a physiological solution.

The observations made by Dr. Mazia on enzyme inactivation are in general agreement with those made by other investigators, namely, that in order to inactivate enzymatic systems, much higher doses of radiation are required than to produce the same effect on chromosomes.

Dr. Mazia: We used 150 kV, Al-filtered, tungsten radiation in these experiments. The films were 20 cm. from the target and radiation was delivered at 13 r per minute. The experiments described by Dr. Goldfeder are very suggestive to workers on chromosomes, because the emphasis on "hit" theories may have overshadowed important indirect effects. The point of emphasis of the experiments on the pepsin-albumin system is that we were able to observe considerable inactivation of the system by doses of less than 100 r. We plan, in this work, to investigate the possibility that the sensitivity of the system is due to the fact that the protein in the films is unfolded, and, therefore, presents much surface for the effect of radiation products in the medium. This investigation would also bear on the "shielding" effects of the components of a mixed system. The pepsin is present in small proportion, but the configuration is such that there is no other protein between it and the aqueous medium.

Dr. C. G. Mackenzie (*Cornell University Medical College New York, N. Y.*): Dr. Mazia, himself, has made it quite clear that the results he has obtained with protein fibers cannot be applied without reservation to chromosomes. Certainly, these experiments represent the gathering of information that is essential for a rational attack on chromosome chemistry, however much more complicated that may prove to be in the light of subsequent work. Dr. Mazia's studies are, therefore, important and stimulating as experiments on chromosome models. But they are of even greater importance in their own right.

Despite the advances resulting from the study of enzymes and substrates in solution, in a homogeneously dispersed system, I am sure all of us are aware of the limitations imposed by such conditions when the results are applied to living systems. In living cells, containing as they do diversified formed elements, reactions must take place not only between molecules in solution but also between molecules and molecular aggregates and between the molecules contained in non-homogeneous solids, that is to say: between the different species of molecules within the formed elements. It is difficult to believe that such is not the case.

Consequently, the fibers studied by Dr. Mazia are far from being just models of chromosomes. They are models of something simpler and, perhaps at our present stage of technical development, of something even more important. They are models of themselves, *of an enzyme and its protein substrate in a solid state.* We now have available a technique for studying the reaction kinetics of such solid enzyme-substrate systems. That, at this higher level of organization, an enzyme-substrate system may exhibit reaction kinetics remarkably different from those it manifests in solution, has already been demonstrated by Dr. Mazia.

I believe that this work represents a revolutionary advance in biochemistry, one that will greatly extend our knowledge of the chemistry of living matter, one that will affect the work of all of us in future years.

PITFALLS IN HISTOCHEMISTRY*

By George Gomori
The Department of Medicine, The University of Chicago, Chicago, Illinois

Although there were a few successful attempts at the chemical identification of microscopic tissue structures as far back as 80 years ago, histochemistry as a systematic science is relatively young. Many of its methods are still imperfect or not sufficiently standardized, the chemical background and meaning of many staining reactions are poorly understood, and the criteria of correct technique and of proper interpretation are not clearly established. It is readily understandable, therefore, that under such conditions some of the methods or the conclusions drawn from the results obtained do not stand up against rigorous criticism. I should like to illuminate this point in connection with four different techniques. They were chosen because loose interpretation of their results has led to conclusions which do not appear to be warranted.

I.

The granules of the enterochromaffin cells (EC) show a number of interesting chemical features which have invited considerable speculation regarding their chemical composition. Their most important chemical reactions are the following:

(1) The chromaffin reaction, shared by the cells of the adrenal medulla, consists in a brown staining of the granules by dichromates. This shade is due to both the reduction of dichromate to CrO_2 and the formation of colored oxidation products.[1] A similar reaction occurs if dichromate is added to an aqueous solution of adrenalin. Using this observation as a starting point, Verne[2] took crystals of a number of related compounds and observed the formation of colors on the addition of dichromate. He found that all o- and p-diphenols and aminophenols are chromffin; *i.e.*, they produce dark brown pigments when treated with dichromate. On the contrary, m-diphenols and -aminophenols gave no reaction.

(2) The argentaffin reaction consists in the reduction of an ammoniacal $AgNO_3$ solution to metallic Ag, with blackening of the granules. It should be made clear, as stressed by Cordier[3] and Hamperl,[4] that the argentaffin reaction is fundamentally different from all other silver impregnations. In the argentaffin reaction, it is a substance contained in the granules themselves that achieves the reduction (Masson-Fontana technique),[5] whereas, in other silver impregnations an extra reducer, applied before or after the silver solution, is responsible for it. Misuse of the term "argentaffin" has resulted in considerable confusion. The argentaffin reaction, if performed correctly, possesses a marked chemical specificity, while silver impregnations in general are not specific in the chemical sense of the word, no matter how selective they may be for certain morphologic structures. Therefore,

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the only silver stains that can be considered selective for argentaffin substances are those which utilize the reducing power of those substances without employing a secondary reducer. Before specificity for any other type of stain can be claimed, it must be shown conclusively by the use of consecutive serial sections that it stains exactly the same cells as the Masson-Fontana technique.

The number of known true argentaffin substances in paraffin-embedded tissues is very limited and includes, besides polyphenols, urates and some poorly known aldehydes. Adrenalin is argentaffin only if the silver solution is applied to fresh tissue. There is no fixative known that will preserve it. The granules of the EC are preserved by all formalin-containing aqueous fixatives but dissolved by all formalin-free aqueous fluids and by alcoholic ones, whether or not they contain formalin.[6, 7] This is an important point, since Dawson[8, 9] and Sharples[10, 11] have used the Bodian method, which involves fixation in an alcoholic medium and the application of a secondary reducer, for the demonstration of the EC. Now, after Bodian's fixative, all the typical reactions of the EC become entirely negative. It is the more remarkable that, in spite of this, Bodian's method will pick out exactly the same cells as the specific stains in the colon and in the small intestine, although in the stomach a number of additional cells are stained. The significance of this fact is not clear, and further investigation is needed for its elucidation. The Popoff[12] stain, on which an original theory on the nature and physiologic role of the EC is based, is entirely nonspecific.

(3) The azo-reaction[13, 14] consists in the production of brilliant reddish azo-dyes when the section is immersed into the alkalized solution of a diazotized amine. This reaction is almost absolutely specific for phenols, and the shade produced depends partly on the nature of the amine used and partly on the complexity of the phenol. The simpler the phenol, the yellower the shade; the more complex, the more purplish the shade.

(4) The indophenol reaction[15] consists in the production of bluish indophenol dyes when the section is treated with dimethyl-p-phenylene-diamine and an oxidant such as NaOCl. In my experience, this reaction is of very limited practical usefulness, at least in vertebrate material, because the shade produced is too pale.

There are a number of other more or less specific reactions described, part of which are poorly understood chemically. Since they are of minor importance, they will not be discussed here.

On the basis of the reactions mentioned, Lison and his coworkers[13, 15] drew the following conclusions: (a) the EC contain some o- or p-diphenol; (b) since the azo-dyes formed are insensitive to alkali, the coupling must have occurred in o-position, which in turn means that the p-position is not free, because whenever that position is free, coupling invariably occurs there; (c) the group attached at the p-position must be relatively simple because the shade of the azo-dyes formed is yellowish. A catechol compound, with a short side chain in p-position, would therefore show all the typical reactions of the EC.

The ideas of the Lison group have been universally accepted and un-

challenged up to this date. There are three weak points in Lison's reasonings, however, which seem to have escaped the attention of subsequent workers.

First of all, the mere fact that the indophenol reaction is positive speaks against a p-substituted phenol, since indophenol dyes, as a rule, do not form unless the p-position is free. Second, derivatives of catechol and hydroquinone, such as adrenalin and homogentisic acid, respectively, are far stronger reducing agents than the granules of the EC. They will reduce an ammoniacal silver solution almost instantly, whereas the granules require several hours. Third, the rule that p-coupled azo-dyes change their shade in alkaline solutions, while those of the o-coupled variety do not, is one to which there are numerous exceptions. Certainly, no positive conclusions can be drawn from the fact that the shade of an azo-dye is resistant to alkali. A number of observations were made recently which cast considerable doubt on the correctness of Lison's ideas.

If Gibbs's reaction,[16] which consists in the formation of blue indophenol dyes when a phenol is added to the alkalized solution of 2,6-dichloroquinone-chloroimine, is applied to sections, a distinct reaction is observed in the granules of the EC. This reaction is negative with catechol and also with any p-substituted phenol.

It can be shown that, under the conditions of histologic technique, the typical reactions of phenols are not the same as they are in the test tube. This difference, according to Coujard,[17] is due to the fact that phenols condense with formaldehyde to form insoluble bakelite-like resins. Therefore, he suggests that, in place of simple test-tube experiments, the conditions of histochemical technique should be duplicated by dissolving the substance in question in serum, writing a mark on a slide, and treating the slide subsequently with all the reagents used in the histologic routine. From the results of reactions applied to such slides, valid histochemical conclusions can be drawn. By the use of this technique, I was surprised to find that resorcinol, although not chromaffin in the test tube, is made just as chromaffin as is catechol. Furthermore, catechol and hydroquinone reduce an alkaline silver nitrate solution within a few minutes, while for resorcinol it takes several hours to do so. The Gibbs reaction, too, is quite intense with resorcinol but negative or negligibly positive with both catechol and hydroquinone. The most striking results, however, are seen with the azo-reaction. Certain diazotized amines will produce various shades when coupled with different phenols, and it is quite obvious that the shades obtained with resorcinol are invariably identical with those shown by the enterochromaffin granules, while the shades obtained with catechol and hydroquinone are utterly different. Therefore, on the basis of these experiments, it would seem that the histochemical reactions of the EC are due to some derivative of resorcinol and not of catechol as it has been believed. This would be quite interesting, since no resorcinol derivative has been known so far to be of physiologic importance in animals.

One more point should be mentioned briefly. Jacobson of Cambridge, England, has adduced excellent evidence[18, 19] to the effect that both EC and

tumors arising from them contain some pteridine. He also makes the statement, however, that the typical reactions of the EC may be due to the presence of pteridines. This is definitely not the case, as can be shown by Coujard's technique. Pteridine does not give a single one of the typical reactions. I was also unable to confirm Jacobson's other contention, that of the presence of desoxyribose in the EC.

The conclusion is that, contrary to previous ideas, the typical histochemical reactions of the EC are due to the presence of a derivative of resorcinol. The presence of pteridine cannot be demonstrated in them with chemical methods now available. They do not contain desoxyribose.

II.

The question of the unity or plurality of phosphatases (Ph) has been a moot point for almost 20 years, with numerous champions on both sides of the fence. There can be no doubt that acid and alkaline Ph are two distinctly different enzymes. However, those who support the theory of plurality find that Ph's are different among themselves in 3 more respects: (1) substrate specificity; (2) organ specificity; and (3) specific activation and inhibition effects. The latter two groups often overlap.

As far as substrate specificity is concerned, it has been established beyond doubt that adenosinetriPh, pyroPh, and hexosediPh are enzymes distinctly different from the nonspecific alkaline Ph. However, Forrai[20] thinks that there is a specific sucrosePh and other sugarPh's; Roche and Latreille[21] maintain that the kidney contains, besides glyceroPh, a phenylPh; and Reis[22, 23] claims the existance of a 5-nucleotidase.

There are data available to the effect that alkaline Ph's of different organs may actually be different enzymes, as shown by their slightly different resistance to inhibitors or by different pH optima. Belfanti and coworkers[24] and Hommerberg[25] believe that bone Ph is different from renal or hepatic Ph. Bodansky[26] thinks that intestinal Ph can be distinguished from bone or renal Ph. Masayama and Shuto[27] found a Ph in hepatomas, different from the enzyme of the normal liver. Cloetens[28, 29] distinguishes two alkaline Ph's on the basis of their different degrees of activation by Mg. Drill, Annegers, and Ivy[30] find that in jaundice a Ph appears in the plasma, different from the normal enzyme in respect to inactivation by cyanide.

The opinions just mentioned represent but a fraction of the literature. Whether the differences reported should be considered as an indication of the existence of several truly different enzymes or as the results of the admixture of various activators and inhibitors or of differences in technique cannot be decided on the basis of our present knowledge. The majority of the experiments were never repeated and the results neither confirmed nor refuted by others than the original authors.

During the last few years, I have prepared a number of highly active purified alkaline Ph extracts from intestinal mucosa, kidney, and bone. The activity of some of the extracts is over 1000 Bodansky units per mg. of N. These enzymes of various sources are entirely indistinguishable from each other in respect to specificity toward 10 different substrates, pH opti-

mum, activation by Mg. or inhibition by cyanide. To put it more exactly, differences between enzymes of different origin are not more significant than those observed between two random batches of, say, intestinal enzyme. However, the yield per Gm. of tissue may show an individual variation of several 100 per cent.

The question is: Can histochemical technique be utilized as an approach to the solution of the problem? A number of papers answer the question in the affirmative (Glick,[31] Dempsey and Singer,[32] and Dempsey and Deane[33]). Because I feel that some of the inferences drawn from the pictures obtained are of doubtful validity, I should like to point out certain sources of error.

First of all, the histochemical demonstration of Ph is a very crude method as far as the quantitative aspect is concerned. It is exceedingly difficult to judge the intensity of the stain. In the majority of instances, Ph occurs highly concentrated within sharply limited areas. If the reaction is positive, one gets a black spot and, since nothing can be blacker than black, the picture may not change appreciably if the slide is incubated for 12 hours instead of 1 hour, except for a slight peripheral extension of the positive areas and some fuzziness of the outlines. Just how much of this is due to diffusion artifacts and how much to additional genuine reaction would be hard to tell.

The pH level at which the slides are incubated has a profound influence on the pictures obtained. At pH 9 the solubility of Ca phosphate is exceedingly low but rapidly increases with lowering of the pH. A precipitate of Ca phosphate will form only if the production of phosphate ions is fast enough to cause local supersaturation in spite of diffusion. This race between the rates of phosphate production and diffusion results in an "all or none" effect, depending on whether the rate of phosphate production is or is not able to overtake that of diffusion to a point where the solubility product of Ca phosphate is exceeded locally. At pH $\pm$8.2, the picture is much less intense than it is at 9, and below pH 7.2 it becomes impossible to produce any precipitate of Ca phosphate, regardless of the length of incubation, in spite of the fact that alkaline Ph possesses considerable activity in that range.

The rate of phosphate formation depends on the original activity of the tissue, on the pH, on the presence of activators and inhibitors, on the permeability of the tissues to the various substrates, and on many other factors not too well understood. Of these factors, the pH of the incubating solution again deserves special attention. Alkaline Ph has an optimal pH between 8.8 and 9.8, depending on the substrate, and activity rapidly declines with the fall in pH. At pH 7 the activity of alkaline Ph is only 3 to 8 per cent of its maximum, again depending on the substrate. Acid Ph, on the other hand, has an optimum around pH 5, but it still possesses some activity at the neutral point.

The rate of diffusion depends on many factors, such as the thickness of the section, the presence or absence of a protective layer of collodion (and on its thickness), the amount of Ca phosphate precipitate already deposited, the temperature of the solution, and random currents in it. This complexity of the situation explains the fact that sometimes serial sections treated

exactly the same way will show noticeably different pictures among themselves, especially in the lower pH ranges, where Ca phosphate is relatively easily soluble and enzymatic activity is low.

It is hard to obtain uniformly satisfactory pictures around the neutral point and hard to interpret them correctly. Since at pH 7 Ca phosphate will not precipitate, lead ions must be used to trap the phosphate liberated. The lead salts of most ester phosphates are practically insoluble at this pH. An exception is glycerophosphate, the lead salt of which is sufficiently soluble to be used in histochemical technique. Unfortunately, this substrate is far from being optimal for acid Ph, as shown by the erratic results obtained if it is used for the *in vitro* determination of acid Ph activity and by the often unsatisfactory results of the histochemical technique for this enzyme. Another complication is the tendency of lead to be adsorbed on various morphological structures at this pH, causing spurious positive reactions, especially when nucleate is used as a substrate.

For the reasons mentioned, pictures obtained around the neutral point should be evaluated with great caution. Unless localizations obtained with various substrates are distinctly different and the results can be duplicated in a number of different specimens (to rule out the effect of a chance ratio between acid and alkaline Ph in the given case), no positive conclusions should be drawn.

At this point, I should like to mention my own experiments. So far, 11 different substrates were tried in the range around pH 9: glycerophosphate, phenylphosphate, phenolphthaleinphosphate, hexosediphosphate, yeast nucleate, thymonucleate, adenosinetriphosphate, aminoethylphosphate, phosphorylcholine, lecithin, and octanoylphosphate. After thorough examination of several hundred slides, each carrying 5 to 15 different tissues, I felt that the pictures obtained with the various substrates are not sufficiently different from each other to warrant any assumption of the existence of substrate-specific alkaline Ph's. At pH 5, only 3 substrates were tried: glycerophosphate, hexosediphosphate, and yeast nucleate. The results with the latter two were not very satisfactory but were good enough to convince me that the differences were insignificant. The same substrates were tried at pH 7, and the results, as I expected, were combination pictures of the typical distribution of acid and alkaline Ph in various ratios.

As for the attempts at selective inhibition of specific Ph's, inactivation by KCN, acid, formaldehyde, and heat were tried. My impression was that these inhibitors lack specificity, although the inhibition may not be entirely uniform because, as a result of the "all or none" effect, areas of low activity may be knocked out altogether, while areas of high activity remain seemingly uninfluenced. If the kidney happens to contain a much higher concentration of the enzyme than the intestine, pictures like those published by Emmel[34] may be obtained. I obtained a number of them. If the kidney is lower in activity than the intestine, however, the reverse effect maybe produced. I have several examples of this in my collection (FIGURE 1).

In summary, it may be said that, so far, the results of histochemical technique do not offer any proof for the existence in animal tissues of phos-

phatases other than the common alkaline and acid enzymes. In plant tissues, however, Glick seems to have proven the presence of substrate-specific acid Ph's, the localization of which is strikingly different.

III.

Bennett,[35] in 1939, described a histochemical method for the demonstration of ketosteroids in the adrenal cortex. Subsequently, his method was utilized for the demonstration of steroid hormones in the ovary (Dempsey and Bassett[36]) and the testis (Pollock[37]). The reaction is based on the formation of yellow phenylhydrazones on treatment of frozen sections with phenylhydrazine. It is prevented by extraction of the lipids with suitable solvents or by treatment with semicarbazide. From these facts Bennett

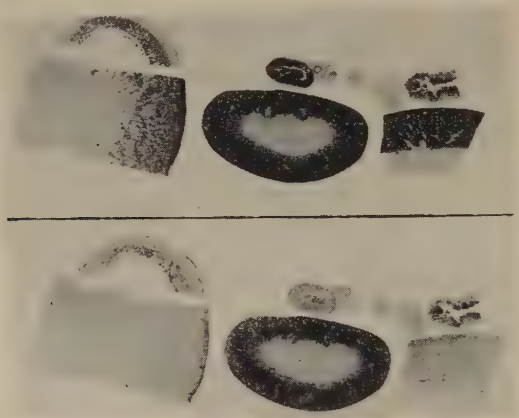

FIGURE 1. Kidney and small intestine of 3 species. Alkaline phosphatase stain. Left to right: dog, rat, rabbit. Upper row: regular phosphatase stain; lower row: N/150 KCN added. In the dog and rabbit, kidney reaction completely abolished while activity in intestine partly preserved; in rat organs, the opposite holds.

drew the conclusion that the reaction is due to the presence of a lipid ketone, since no lipid aldehydes have been detected in the adrenal cortex.

The serial publications of the Feulgen[38, 39, 40] group on plasmalogen, a cyclic acetal which occurs in large amounts in almost all lipid-containing tissues and which on hydrolysis yields plasmal, a mixture of stearic and palmitic aldehydes, must have escaped Bennett's attention.

In 1942, it was shown[41] that the plasmal reaction, produced with Schiff's reagent, shows a localization invariably and exactly identical with that of the phenylhydrazone test. The opinion was voiced that the latter is not specific for ketosteroids at all but demonstrates an overwhelming bulk of plasmal. It is positive for ketosteroids, too, which plasmal, at certain specific sites, may or may not contain in infinitesimal amounts. The following evidence was adduced:

(1) In both plasmal and phenylhydrazone techniques, the use of an oxidizer (or some hydrolytic catalyst) is necessary before the application of the

reagent proper. The intensity of the stain is, within limits, proportional to the length of pretreatment. In general, it appears that phospholipids require less oxidation than simple neutral fats. Without oxidation both reactions are entirely negative. This is understandable in the case of plasmal, since the acetal linkages must be broken first. But there is no reason why it should be necessary in the case of ketosteroids, which ought to give a prompt reaction without any pretreatment. Therefore, the phenylhydrazone reaction can be accepted as a presumptive evidence for the presence of ketosteroids only if it is positive without any oxydative or hydrolytic pretreatment.

(2) Very intense plasmal and phenylhydrazone reactions were obtained in tissues such as necrobiotic tumors of all kinds, tubercles, brain and peripheral nerves (in fact, Verne[42] suggests the plasmal reaction as a stain for myelin sheaths), and even in adipose tissue. None of these tissues are known to contain ketosteroids.

Dempsey and Wislocki,[43] although acknowledging the fact that the phenylhydrazone test is not capable of differentiating between aldehydes and ketosteroids, still feel that it is reasonable to attribute a positive reaction to the hormones or their precursors. Their main argument is that both the plasmal and the phenylhydrazone reaction are positive at all sites where ketosteroids are known to occur. This is, no doubt, true; but it would hold for a simple fat stain just as well, since all sites where steroid hormones occur are sudan-positive. In fact, the reactions in question are actually not much more specific for ketosteroids than any fat stain. Verne[44] has shown that such pure fats as triolein, butter, or lecithin give typical aldehyde reactions after mild oxidation.

On the basis of the foregoing, all specificity of the phenylhydrazone reaction for ketosteroids must be denied. Why these hormones occur at sites rich in phospholipids is a different problem which deserves attention.

IV.

Oster and Schlossman have described a histochemical method[45] for the demonstration of amine oxidase. They incubate frozen sections with a buffered solution of tyramine. The formation of aldehyde is shown by the appearance of a purplish-blue shade when the sections are immersed in Schiff's reagent, fuchsine-sulfurous acid. However, since tissues contain various amounts of plasmalogen, which under the influence of mild oxidants, even air, breaks down to form plasmal, an aldehydic compound, it is necessary first to transform plasmal into a non-reacting form to avoid its being mistaken for newly formed aldehyde. This is accomplished in the following way: First, the sections are treated with a 2 per cent solution of $NaHSO_3$ at 37°C. for 24 hours. The bisulfite addition compound of plasmal does not react with Schiff's reagent any more, and the sections, after thorough washing, are ready for incubation with tyramine. Identical reactions are obtained when the sections are incubated with tyrosine. The authors explain this by enzymatic decarboxylation followed by oxidation of the amine thus formed. In fact, there is some reaction obtained even in sections incubated

without any substrate added, owing to the enzymatic breakdown of native substrates.

When this method is analyzed critically, its theoretical background shows a number of weak points:

(1) The oxidation of tyramine by amine oxidase usually goes beyond the aldehyde phase unless the aldehyde formed is trapped (*e.g.* by semicarbazide), and the final product is oxyphenylacetic acid.[46, 47] But, even if the process is stopped at the aldehyde stage, it is unlikely that oxyphenylacetaldehyde, being fairly soluble, would remain *in situ*.

(2) The fact that an identical reaction is obtained with tyrosine as a substrate is very hard to understand, unless it is assumed that the rate of amine oxidation is much faster than that of decarboxylation, and that the amine formed is immediately oxidized. Otherwise, it would diffuse out into the medium, and its local concentration would be exceedingly low. It is possible that such a great difference in the velocities of the two reactions exists; but, so far, there is no evidence for it.

(3) The addition compounds between bisulfite and aldehydes, especially of the higher terms, are very unstable and prone to dissociate into aldehyde and bisulfite. It is unlikely that plasmal-bisulfite would stand up against incubation at 37°C. for 24 hours.

To test the validity of these objections, the following experiments were performed:

(1) A guinea-pig kidney mash was incubated with buffered M/50 tyramine for 24 hours, and a control was run without tyramine. The deproteinized filtrates were both Schiff-negative, showing that no aldehyde was formed.

(2) Frozen sections of guinea-pig kidney were treated with bisulfite for 24 hours and washed thoroughly. They were incubated for 24 hours with tyramine and with buffer solution alone. In addition, several sections were immersed for 10 minutes in 2 per cent trichloroacetic acid, thoroughly washed, and incubated with tyramine. After incubation, all sections were immersed in Schiff's reagent, where all of them started to get bluish after about 10 minutes and were quite blue after 45 minutes. Slices incubated with or without tyramine or after trichloroacetic acid treatment did not show any appreciable difference in the intensity of the reaction. Moreover, the distribution of the blue staining areas under the microscope could not be distinguished from the regular pattern of the distribution of plasmal, althought the shade was different. It should be remarked here that no reaction is obtained with Oster and Schlossman's technique if liver sections are used,[48] although liver has a high amine oxidase activity. This negative result may be due to the fact that liver contains practically no plasmal.[44]

(3) The color reaction of 4 different aromatic aldehydes with Schiff's reagent was tested. Unfortunately, p-oxyphenylacetaldehyde was not available. The following reactions were obtained: benzaldehyde, purple, shade indistinguishable from that of plasmal; anisaldehyde, definitely more reddish; vanillin, rose-red; p-dimethylaminobenzaldehyde, scarlet. None of the aldehydes showed the blue shade of the sections.

On the basis of these results, it would seem that Oster and Schlossman's

technique does not demonstrate amine oxidase. The blue shade may be due to some alteration in the plasmal molecule, regenerated from its bisulfite compound, but the exact chemistry of this process is not clear. It may have something to do with the pH of the bisulfite solution. A fresh solution of bisulfite has a pH of around 5, but at 37°C. it may turn very acid (pH 2.4 to 2.6) within a few hours. Some solutions, however, for a reason not clearly understood, will show very little lowering of the original pH. This unpredictable behavior of the bisulfite solution may explain the poor reproducibility of the results, admitted by Oster and found by me.

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Discussion of the Paper

H. Waelsch (*Columbia University and New York State Psychiatric Institute and Hospital*): Results obtained in determining the higher fatty aldehydes (plasmal) with the aid of the Schiff reagent have to be accepted with caution, since it can easily be shown that the age of the preparation and the accompanying lipids influence the values decisively. Lecithin, after 5 recrystallizations as the cadmium complex (phosphorus-choline ratio 1:1),

gave, after 4 days over sodium hydroxide at 15 mm. pressure, a fuchsin color value corresponding to 70 gamma palmitaldehyde; after 8 days, a value of 185 gamma; and, after 14 days, one of 235 gamma per 100 mg. This increase in apparent aldehyde content may be related to an oxidation of the unsaturated fatty acids and may simulate, in a preparation of not well-defined history, a high original aldehyde content.

On the other hand, if one carries out the Feulgen test with synthetic aldehydes or acetals in the presence of lecithin or synthetic non-ionic detergents, the color values may be suppressed down to a few per cent of the control sample.

Dr. G. Gomori: No doubt the age of the tissue is an important factor in the intensity and the extent of both the plasmal and the phenylhydrazone reaction. Frozen sections which are kept floating, unprotected from air for a few days, will become increasingly positive for plasmal. All rancid fats are also extremely strongly positive.

M. A. Lessler(*Department of Biology, New York University, New York*): There is need for a critical re-evaluation of the specificity of both the plasmal and the Feulgen reactions. Although the same reagent is used in both (leucofuchsin, produced by treatment of basic fuchsin with an excess of sulphurous acid), the Feulgen reaction involves a preliminary mild acid hydrolysis which is not used in the plasmal reaction.

Is it not true that we should interpret the plasmal reaction cautiously, since it is simply an application of the Schiff reaction for aldehydes to tissue sections? This reaction has been shown to be non-specific by Lison (Bull. d'Hist. Appliquée **9:** 177. 1932) and others. Leucofuchsin can be recolorized on reaction with basic compounds such as the purines, pyridine, and methyl ketones and other naturally occurring intracellular substances such as starch, glycogen, and other polysaccharides. Treatment of the sections with aqueous and then with alcoholic solutions removes certain soluble substances, but even after such extraction there is still present in some cells a residue which will regenerate the color of the leucofuchsin. Therefore, conclusions as to the nature of the residue cannot be made unless other techniques are used for the purpose of eliminating the possibility of several compounds entering into the reaction.

On the other hand, the Feulgen reaction can be controlled by use of sections which are not subjected to the mild acid hydrolysis. These should be completely negative to the leucofuchsin reagent. If the controls stain, they are showing the non-specific plasmal reaction. The sections subjected to the Feulgen reaction should be interpreted only in the light of the controls.

With adequate control, the Feulgen technique is uniformly specific for nuclear material. The interpretation that this technique stains the aldehydes formed by the mild acid hydrolysis of thymonucleic acid is open to some question at present. Stedman and Stedman (Nature **152:** 267. 1943) and Carr (Nature **156:** 143. 1945) have raised objections against the specificity of the Feulgen reaction. These have been refuted by Stowell (Stain Tech. **21:** 137. 1946) and Dodson (Stain Tech. **21:** 103. 1946).

Interpretations of the Feulgen reaction are of basic importance for an

understanding of the chemistry of tissues. A reinvestigation of the specificity of this reaction is being conducted in our laboratory.

Dr. G. Gomori: The plasmal reaction has a limited specificity but, if it is performed correctly, there is little interference on the part of non-aldehydic substances. Uric acid and saccharides, for instance, do not recolorize leucofuchsin under the conditions of the test. In addition, just as non-hydrolyzed sections can serve as controls for the Feulgen test, sections treated with semicarbazide or, preferably, with phenylhydrazine can be used as controls for the plasmal reaction.

Dr. H. Stanley Bennett (*Massachusetts Institute of Technology, Cambridge, Mass.*): Since Dr. Gomori has chosen to discuss the phenylhydrazine reaction, which I applied to the adrenal gland, it is appropriate that I comment on his paper.

First, I might stress that I made no claims that the reaction I used was one which demonstrated the presence of ketosteroids. In my paper, I stated clearly that all the reactions I used were characteristic of aldehydes and ketones in general, and that none of them was specific for the adrenal cortical sterones. Hence, Dr. Gomori and I have no disagreement with respect to the chemical significance of the phenylhydrazine reaction.

Second, with respect to the Feulgen plasmal reaction, it is well known that the recolorization of leucofuchsin, or Schiff's reagent, is by no means specific for aldehydes. Lison has shown that Schiff's reagent can be recolorized by ketones, aldehydes, and other reducing groups. Hence the reaction Dr. Gomori used can be produced by ketones as well as by aldehydes.

Third, I was not aware that oxidation of tissues prior to phenylhydrazine treatment had any role in bringing out the reaction. Iodine, molecular oxygen in alkaline buffer, and indophenol were all used in my hands with a view toward eliminating ascorbic acid, which might give a false positive with phenylhydrazine. Dr. Gomori has pointed out that iodine or molecular oxygen might be responsible for bringing out the so-called "plasmal reaction." He made no mention of indophenol, although I reported that the phenylhydrazine reaction occurred after the tissues had been treated by indophenol. I should like to ask Dr. Gomori if he knows of any evidence that indophenol can bring out the plasmal reaction.

[Dr. Gomori made remarks to the effect that he knew of no such evidence.]

Fourth, with respect to the "plasmal reaction," Feulgen and Verne and their coworkers presented no conclusive evidence that the compounds they were dealing with were aldehydes and not ketones, as the reagents they used—Schiff's reagent, phenylhydrazine, semicarbazide, and thiosemicarbazide—are reagents for ketones as well as for aldehydes. The "plasmal reaction" can, hence, be produced by aliphatic ketones as well as by aliphatic aldehydes, and in this connection it is worth mentioning that the corticosterones are all aliphatic ketones.

Fifth, in spite of the fact that the phenylhydrazine reaction is not specific for the ketosteroids, but shows the presence of aldehydes and ketones of whatever nature, it is still, in my opinion, of localizing value in tissues under some circumstances. For instance, in the adrenal cortex of the cat, the

reaction is not present throughout the width of the cortex, but is present in a narrow band corresponding to the zone of spongiocytes. Cortical cells peripheral to this zone show no detectable yellow and, in the inner zones, the reaction is likewise absent or, in some cases, very weak. Hence, within the limits of sensitivity of this method, acetone-soluble, water-insoluble aldehydes and ketones of any sort cannot be detected in those cortical areas where the reaction is absent. Thus, it follows that the cortical sterones can be regarded as confined to and localized in some or all of those areas where the reaction is positive and absent elsewhere in detectable amounts. In the human, dog, and rat, the phenylhydrazine reaction occurs throughout the width of the cortex. In such cases, the reaction does not indicate any differential localization of ketones and aldehydes within the various zones of the cortex. Likewise, in the testis, Pollock showed the phenylhydrazine reaction to be positive in the interstitial cells and absent from the tubules. It follows that acetone-soluble, water-insoluble compounds with the carbonyl group (such as testosterone) are confined to the interstitial cells, which, hence, appear as a site of possible localization of any ketosteroids (such as testoreone) which may be in the testis, whereas the tubules do not give evidence of being such a site of localization.

Now, perhaps, the area of disagreement between Dr. Gomori and myself is not as great as it may have seemed originally.

ENZYME SYSTEMS OF ISOLATED CELL NUCLEI

By Alexander L. Dounce
School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

Introduction

The preparation of nuclei on a large scale by chemical or physicochemical methods probably dates back to the work of Miescher, who isolated pus cell nuclei by a rather drastic method which included the use of a pepsin-hydrochloric acid mixture to digest away the cytoplasm. Miescher and Kossel also obtained nuclei from fish spermatozoa which were prepared by a rather mild procedure which would have permitted a subsequent study of enzymes in the isolated nuclei. However, at the time this work was accomplished, enzyme chemistry had not been developed to an appreciable extent. Much useful work might be carried out on enzyme studies of fish spermatozoa by any one having an available source of material.

Subsequent workers prepared bird erythrocyte nuclei by methods which would have permitted enzyme studies, but such studies were not made. For example, preparations of bird erythrocyte nuclei liberated by freezing and thawing were made by Warburg. References to this early work and to much of the later work can be found in a review article on gene structure and action by A. Gulick.[1]

Recent Methods for Preparing Isolated Cell Nuclei

In more recent times, nuclei have been made from mammalian spermatozoa by means of sonic disintegration of the cells. The nucleated halves of sea-urchin eggs were prepared by Harvey, using a centrifugation technique. Cytochrome oxidase was found in the nuclei in both cases. These results have been discussed briefly by Dounce.[2]

Bird erythrocyte nuclei have been prepared by Yakushizi,[3] Laskowski,[4, 5] and Lan and Dounce.[6] The latter authors used the same method previously employed by Yakushizi,[3] but failed to realize it because they had not obtained the original paper, which was published in a Korean journal. We should like to acknowledge this oversight at the present time. Yakushizi also worked with pus cell nuclei.[7]

Although the bird erythrocyte nuclei obtainable by the most recent methods are admirably suited for enzyme studies, as far as mildness of the method of preparation is concerned, very little work of this nature has been carried out. Dounce and Seibel found acid phosphatase present in chicken erythrocyte nuclei.[8] The rate of oxygen consumption and anaerobic glycolysis of chicken erythrocyte nuclei has been studied by Hunter and Baufield.[9]

In the case of tissues of mammalian origin, two methods have been published in detail which have been used to obtain nuclei suitable for enzymatic study. One of the methods is that of Behrens.[10–15] His procedure consists of drying the tissue in question in the frozen state, then cutting it up and pulverizing it in a ball mill. The pulverized material is sifted, repulverized,

and, finally, the nuclei are obtained by repeated centrifugation from a mixture of benzene and carbon tetrachloride. Behrens found little lipase in nuclei prepared in this manner, but considerable arginase.

The method of Behrens is, according to the numerous publications of the author, quite general and has been applied to plant tissue as well as to a number of animal tissues. It has been employed in a modified form by R. Williams *et al.*[16] to compare the vitamin content of nuclei from normal tissue and tumor tissue. The results obtained in this study are shown in TABLE 1. Since the assays were microbiological for the most part, it is difficult to state whether the vitamins were measured as such, or as parts of coenzymes. The method of Behrens, in a slightly modified form, also has been used by Mayer and Gulick[17] to study the protein composition of nuclei.

It seems probable that the method of Behrens is the best procedure available at the present time for preparing in quantity nuclei upon which studies

TABLE 1
VITAMINS IN NUCLEI
(Micrograms of Vitamin Per Gram Dry Material)

Vitamin	*Whole beef heart*	*Beef heart nuclei*	*Whole mouse cancer*	*Mouse cancer nuclei*
Riboflavin	34	130	8.3	7.0
Inositol	7600	2000	450	400
Nicotinic Acid	320	900	130	95
Panthothenic Acid	75	270	60	43
Thiamin	32	90	9.0	7.4
Pyridoxin	4.4	4.2	0.87	0.90
Folic Acid	1.1	3.9	17	13
Biotin	0.52	0.25	0.35	0.27

are to be made of water-soluble constituents such as coenzymes and low molecular weight metabolites, because the use of an aqueous medium is altogether avoided. Since no photographs have yet been published of nuclei obtained in this way, the state of purity is not clear, although analyses for desoxyribonucleic acid indicate a reasonable degree of purity. The method is, however, laborious, time-consuming, and not well suited for preparing nuclei for studies of delicate enzymes which might be destroyed or considerably damaged by the procedures involved.

The second method described in detail which has been used to prepare on a large scale nuclei suitable for enzyme studies is the method of Dounce.[2] This method is based upon the earlier citric acid methods employed by Stoneburg,[18] Marshak,[19, 20] and Haven and Levy,[21] but far less citric acid is employed. The pH is maintained at approximately 6.0, where nearly all enzymes are perfectly stable, particularly in the cold. This method has been used by Lan[22, 23] in this laboratory to study enzymes of isolated liver-cell nuclei. Lipid studies have also been made on nuclei prepared in this way by Dounce[24] and by Williams *et al.*[25] Dounce[26] has determined desoxyribonucleic acid in nuclei prepared in the same manner. Hoerr has used

the method to obtain nuclei from liver cells.[27] The method has been used in a slightly modified form by von Euler and co-workers[28–32] to study nuclear enzymatic activity, but, more particularly, to study nucleic acid and nucleoproteins of the nuclei.

It is our opinion that, for gross analytical purposes where it is not necessary to maintain enzymatic activity, it may be best to prepare cell nuclei at pH 3.8 to 4 whenever possible.[24, 26] Although many enzymes may be denatured at this pH, there should be maximal retention of lipid, nucleic acid, and histone, and protein also should be quite well retained.

Since nuclei prepared at pH 4 or at pH 6, as described, may lose low molecular weight, water-soluble components such as coenzymes and substrates, if these materials were present when the nucleus was in the intact cell, material of this sort must be added when necessary in investigating enzyme systems of the isolated nuclei. Thus, the method of Dounce may not be suitable for preparing nuclei in which coenzymes, vitamins, or low molecular weight, water-soluble substrates are to be studied, and should not be used for studies of this kind, unless it can be proved that such constituents are not extracted from the nuclei during their isolation.

This statement applies to any method for preparing nuclei in which an aqueous medium is employed, with or without the addition of acid. However, in enzyme studies the principal object, at least at the outset, is to preserve the apoenzymes in an undamaged state. This appears to be accomplished by the method of Dounce.

Recently, the method for preparing nuclei at pH 6.0 has been improved in this laboratory by reversing the first steps of the procedure and using temperatures close to zero throughout the preparation. The latter procedure has been made possible by the acquisition of a refrigerated centrifuge.

Other workers have reported the separation of cell nuclei at zero degrees centigrade without the use of any citric acid for lowering the pH[33–35]. However, since no photographs have been published which show enough nuclei to enable one to get an idea of the state of purity of the preparation, we cannot easily compare such preparations with our own at the present time. We have never been successful in preparing good samples of nuclei without the eventual use of acid to lower the pH to 6.0, and we have found that somewhat better results are obtained with citric acid than with acetic acid.

In the improved procedure, 200 grams of frozen liver, after being cut into small pieces, are mixed in a previously cooled Waring Blendor, with 350 ml. of ice water containing 50 grams of crushed ice, for 45 seconds to one minute before adding any acid. Then enough 0.1 molar citric acid is added dropwise to lower the pH to a value between 5.8 and 6.0. We have found that 8 to 9 cc. of 0.1 molar citric acid are generally sufficient for this purpose, which amounts to 80 or 90 per cent of the quantity of citric acid used in the original method. Possibly the preliminary mixing of the liver in the blendor with ice water causes the liberation of a slight amount of acid.

The blendor is then allowed to run for 15 minutes. Three 50-gram portions of ice are added from time to time to keep the temperature as low as possible. The material is then strained through cheesecloth and the nuclei

are isolated as previously described.[2] If possible, all operations should be carried out in a cold room so that the temperature remains between zero and 5 degrees C. throughout the preparation. It is possible and perhaps advantageous to use 50-gram portions of liver instead of 100-gram portions, and to halve the amounts of all other constituents added. In this case, the total time of blending is reduced to 8 minutes.

The Waring Blendor used in this work has been model 14, which uses two amperes of current. Recently, we have found that the new model 15 Waring Blendor, drawing three amperes, destroys nuclei from liver cells. This blendor can be used, however, in conjunction with a rheostat which reduces the operating voltage to about 95 volts.

The improved method appears to eliminate an objection raised by Dr. Claude in a private communication. He thought that possibly in the original method only acid-fixed nuclei were being isolated, that is, nuclei which had been subjected to a low pH when the first few pieces of liver were added to the cold citric acid in the blendor. We had found, however, that the pH rises so rapidly after the addition of small amounts of liver that such an assumption was improbable. Nevertheless, the fact remained that some of the isolated nuclei had been subjected to lower pH than others, and hence the improved method is of distinct advantage.

One might inquire why we are so particular about the pH employed during the preparation of isolated nuclei. The principal aim in this respect was to use a pH as close to 7.0 as possible. It was found that between pH 4 and 6 an agglutination of cytoplasmic granules occurred to such an extent that separation of nuclei from the agglutinated mass was very difficult or impossible. At pH 4, the granules became dispersed sufficiently so that nuclei could easily be isolated. However, this pH is too low for sensitive enzymes. At pH 5.8 to 6.0, the cytoplasmic granules again became dispersed and isolation of nuclei was possible. If the pH was raised to 6.5, however, the nuclei disappeared during the process of centrifugation, possibly because of the action of cytoplasmic enzymes. Although at the present time most of our operations are carried out at zero degrees C., the temperature in the Waring Blendor may rise to 5° or 6° C. during the last few minutes of blending, and it might be at this point that the nuclei are dissolved.

It has been found that the addition of salts or buffers prevents proper breakdown of the cells at pH 6.0, so that these materials must be avoided if our method is being employed. It was found, furthermore, that isotonic saline could remove protein from isolated liver-cell nuclei to a greater extent than distilled water, which apparently removes very little.[2]

The use of protein solutions to prevent unwanted swelling of nuclei with subsequent extraction of nucleoprotein at pH values above 7.5 has been studied by von Euler.[29] It is not yet known whether this procedure can be applied to the preparation of nuclei for enzyme work.

The yield of nuclei prepared from liver cells by our improved method described above is between two and three grams per 100 grams of liver. The method has been designed to obtain as pure nuclei as possible rather than to obtain all the nuclei present.

It is an interesting question as to what factors are responsible for the liberation of nuclei from the cells in the methods involving aqueous media. It is certain that strong citric acid has a pronounced action in disrupting the cell membrane[36] and perhaps, also, the cytoplasm. At pH 6.0, where very little citric acid is used, this effect is much lessened, but appears still to be operative, since, as has been stated, citric acid is slightly better than acetic acid in preparing nuclei at pH 6.0. Part of the effect of the acid may be a pH effect and part an anion effect, possibly due to a complexing of magnesium and calcium which are found in the cell wall.[37]

Dr. Mirsky has pointed out in the discussion that the shearing effect of the Waring Blendor is of great importance in preparing nuclei. This shearing effect can be increased to a point where the nuclei themselves are completely disrupted, simply by using much less material so that the blendor can operate at higher speed. The model 15 Waring Blendor, when used without a rheostat, apparently disrupts liver-cell nuclei because of excessive speed.

Another factor which may aid in breaking the cells and liberating nuclei is a very slight autolytic action. We ordinarily freeze the liver before using it by placing it in the unit of a refrigerator. In this manner, the freezing is not instantaneous. When livers were frozen quickly by cooling in a calcium chloride-ice bath or in an acetone-dry ice bath, the cells were much more resistant, and it became difficult to obtain nuclei by our procedure. It is also possible that slow freezing might tend to disrupt the cell by formation of ice crystals.

Schneider has reported the preparations of nuclei by using the Potter-Elvehjem ground glass homogenizer on small samples instead of using the Waring Blendor.[34, 35] We have been able to obtain quite clean preparations of liver-cell nuclei by use of small samples with a ground glass homogenizer. Our homogenizer, which is of somewhat different design from the one originally employed by Potter and Elvehjem, is shown in FIGURE 1. The homogenizer is run from a motor, and an ice-water bath in used for cooling. The length of time necessary to break the cells will depend upon the speed of the motor and can be found by trial and error. The frozen liver is cut up into fine pieces before being added to the homogenizer. The outside portion of the homogenizer is raised and lowered, the plunger being held at a fixed level, in order to make certain that all pieces of the liver get into the bottom and become ground. If the homogenizer is run too long, there is a tendency to produce "eviscerated" nuclei which have been deprived of their chromatin. Such nuclei are perhaps in reality only nuclear membranes.

It has been possible to apply our improved method of preparing nuclei to sheep, dog, and human kidney and to sheep pancreas, using some modifications which are necessary to remove fiber. This work will be described elsewhere. Thus far, we have not been successful with thymus or spleen. However, von Euler prepared nuclei from thymus and other tissues[28, 29] by a modification of our original method, which involves a short exposure of the nuclei to pH 4.0 at zero degrees C., followed by isolation of the liberated nuclei at higher pH values.

We have found that tumor tissue is likely to be much more resistant than liver or kidney, as far as breaking the cells is concerned. It has been impossible, thus far, to obtain very good nuclei from Hepatoma 31 or from Walker Carcinoma 256 without the use of strong citric acid. This difficulty with tumor tissue also has been noted by von Euler.[29] We did succeed however in making a fairly good preparation of nuclei from a mouse leukemia.

In concluding the discussion of methods of preparing isolated nuclei by

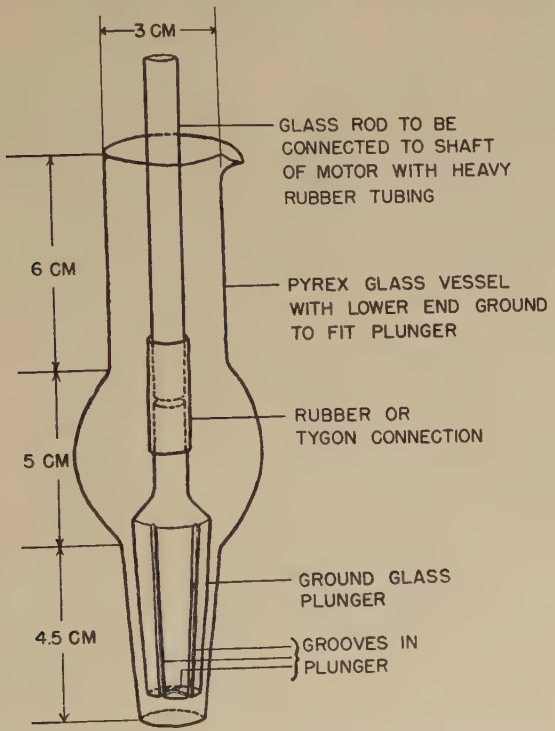

FIGURE 1. Ground glass homogenizer. Smaller models also can be used.

physicochemical methods, it should be stated that it is not enough to observe the liberation of some nuclei microscopically. It is necessary to break a very high proportion of the total cellular material present and subsequently to remove quite completely any remaining whole cells and, also, to remove the various cellular granules. It appears to us that articles on new methods of isolating cell nuclei should include photomicrographs in order that the reader may obtain some idea of the state of purity of the nuclei.

Purpose of Studies of Isolated Cell Nuclei

Before stating the results of enzyme studies of isolated cell nuclei, up to the present time, we should like to digress long enough to outline briefly

two hypotheses which are, at present, directing our general research aims and thinking. We are motivated by the hope of discovering something fundamental about cell division which can be utilized to throw light on the abnormal cellular growths which we call cancer. In understanding the phenomenon of cancer, a knowledge of the synthesis of protein and nucleic acid becomes important, as well as knowledge of sites where these syntheses occur.

It appears possible for nucleic acid and protein to be synthesized in the cytoplasm without cell division's taking place, as is evidenced by work on viruses and by the work of Spiegelman *et al.*, on the so-called plasma genes.[38] References will be found in Spiegelman's article to other work supporting this view. It is, furthermore, indirectly supported by the work of Brues, Tracy, and Cohn.[39] However, when cell division takes place in mammalian tissues, the nucleus participates and nuclear material as well as cytoplasmic material is synthesized. It is not implied that the nucleus never carries on metabolism unless the cell is dividing. Indeed, the contrary must be true at least in special cases, as evidenced by the work of Duryee reported in this monograph, and by indirect evidence such as that collected by Stern.[40] The interesting work of Brues, Tracy, and Cohn[39] on the uptake of radioactive phosphate by desoxyribo- and ribonucleoprotein indicates that, in liver cells, the nucleic metabolism is low in the nucleus in the resting cell and much higher in the dividing cell, whereas the nucleic acid metabolism in the cytoplasm, in either the resting or dividing cell, is considerable. Regenerating liver and tumor were used as sources of dividing cells, and nuclei were isolated by the use of strong citric acid in this work.

It occurred to us, after finding a number of enzymes present in the nucleus of the liver cell, that an important factor limiting metabolism in the nucleus of a resting cell might be the non-availability of certain important metabolites in the nucleus rather than a lack of enzymes. These metabolites might be low or absent because of their being screened out by the cytoplasm, after entering the cell. Such hypothetical screening could depend upon the presence of active enzyme systems in the cytoplasm capable of very rapidly transforming incoming metabolites into products not useful for the synthesis of protoplasm. For example, glucose might be rapidly turned into glycogen and deposited in an insoluble form, while amino acids might be rapidly deaminated and subsequently oxidized or converted to carbohydrate or ketone bodies; or they might be used up for protein synthesis in the cytoplasm.

According to this hypothesis, any factor which would permit metabolites necessary for protoplasmic synthesis to penetrate through the cytoplasm to the nucleus should favor cellular division. In the case of regenerating liver, the situation might be that of flooding the remaining liver with metabolites (because of the increased rate of blood flow) to such an extent that the capacity of the cytoplasm to assimilate the incoming material would be exceeded, with the resulting penetration of the metabolites to the nucleus. It seems possible that any factor directly or indirectly damaging cytoplasm might eventually reduce its metabolic capacity to the point where metab-

olites necessary for protoplasmic synthesis could penetrate to the nucleus. Such a cytoplasmic derangement, if permanent, would be classed as cancer. Other investigators[41, 42] have previously considered cancer as being primarily a disease of cytoplasm. It is even possible that an unfavorable mutation might indirectly cause cytoplasmic damage. Of course, obvious morphological nuclear derangements in cancer cells are well known, but it is possible that the cells which have nuclear derangements do not maintain themselves.

The cytoplasmic screening hypothesis just outlined is to be applied only to cells capable of division under the proper conditions. One piece of evidence which to some extent supports the hypothesis is as follows: It has been found by Chipps and Duff[43] that glycogen appears in the nuclei of liver cells in uncontrolled diabetes and in some other pathological conditions, whereas it is absent normally from the nucleus. We have found no glycogen in nuclei obtained from normal rat liver.[24] In diabetes, the excessive gluconeogenesis occurring in the liver, presumably in the cytoplasm of the cells, might well flood the cell with glucose to such an extent that this metabolite could penetrate to the nucleus and there become transformed to glycogen. We have recently found phosphorylase activity in nuclei isolated from rat-liver cells, and this finding appears to strengthen the above evidence.

It is possible that the hypothesis of cytoplasmic screening can be further investigated experimentally by comparing the concentrations of other substrates in isolated nuclei with their corresponding concentrations in cytoplasm or whole tissue. The method of Behrens is probably the only method available, at the present time, which is certain to leave water-soluble substrates in the nucleus, and which is therefore suitable for this purpose. For studying most substrates, microdissection probably would not yield enough material, although it might be very helpful in some cases.

A second general aim in studying isolated cell nuclei was developed as a result of Greenstein's conclusions[44] about the enzymatic pattern of malignant tumor cells. Greenstein has shown that tumors tend to exhibit a constant enzyme pattern. A given enzyme may be higher or lower in concentration in a tumor than in the tissue from which the tumor is derived, but the final enzyme pattern is reasonably similar for the various tumors studied. It is the rule, although there are exceptions, for cancer tissue to lose specialized metabolic function. That is, the cancer cell rarely serves the metabolism of the entire host, but instead serves only its own metabolism, which is the metabolism necessary for the synthesis of protoplasm.

It occurred to us that the main enzyme systems present in the nucleus might be those necessary for the synthesis of protoplasm, and that, in cancer, the enzyme pattern of the cytoplasm might revert to the enzyme pattern of the nucleus, since here, in both the nucleus and the cytoplasm, the chief function seems to be to synthesize protoplasm rather than to carry out specialized metabolic activities for the benefit of the organism as a whole.

If this idea is correct, the enzyme pattern of nuclei from ordinary resting cells should resemble the total enzyme pattern of cancer, which would be

similar in nucleus and cytoplasm. An investigation of this point is now under way, and in the following section it will be seen to what extent the enzyme pattern thus far obtained for isolated nuclei resembles the enzyme pattern of tumors.

These two hypotheses have been presented to show our general aims, and, also, to try to interest other workers in this field of research. Even if both hypotheses eventually should prove to be false, they may yet serve a useful purpose in promoting systematic work on cell nuclei.

TABLE 2
ENZYMES IN ISOLATED RAT-LIVER CELL NUCLEI AND RAT HEPATOMAS

Enzyme	*Concentration in nucleus as per cent of concentration in whole tissue*	*Concentration in rat hepatoma 31 as per cent of concentration in normal tissue*
*Aldolase	40	—
†d-Amino oxidase	100	10
*Arginase	113	10
‡Catalase	0.05–0.1	0.1
†Choline oxidase	0.0	0.0
Cytochrome oxidase	50–60	28
Cytochrome C	low	**22
*Enolase	50	—
Esterase	50	lowered
Alkaline phosphatase	192	13,550
Acid phosphatase	25–30 (minimal)	200
*Phosphorylase	66 after grinding 26 without grinding	— —
*Lactic dehydrogenase	40	present
*Succinic dehydrogenase	0.0	††14
†Uricase	100 (minimal)	5

* Nuclei prepared by the improved method. ** Primary tumor. ‡ See footnote p. 991.
† Work of T. H. Lan. †† Mouse hepatoma.

Results of Investigations on Enzyme Systems of Isolated Liver Cell Nuclei

References to earlier work on the enzyme content of isolated cell nuclei are given in the paper of Dounce.[2] More recently, Dounce and Lan[22–24] investigated a number of enzymes in liver-cell nuclei isolated by the original method of Dounce.[2] The results of these investigations, with additional results on the enzymes aldolase, enolase, lactic dehydrogenase, and phosphorylase are shown in TABLE 2. The enzymes appear to fall into three classes. The first class comprises the enzymes arginase, d-amino oxidase, alkaline phosphatase, and uricase, whose concentrations appear to be as high or higher in the isolated nuclei than in whole liver. The second class comprises the majority of the enzymes investigated, whose concentrations were found to be roughly one half their concentrations in whole liver.

The most remarkable of the results thus far obtained, however, appears to us to be that the enzymes catalase, succinic dehydrogenase, and choline oxidase, which fall into a third class, are very low in concentrations in the nuclei as compared to their concentrations in the whole liver. Cytochrome

C was also low in concentration in the nuclei.* Since the nucleus of the liver cell constitutes only about 8 per cent of the cell volume,[20] the concentration of an enzyme in whole tissue can be taken as roughly equal to its concentration in cytoplasm.

Succinic dehydrogenase is of particular interest because of its role in the carbohydrate oxidation cycle. In the case of succinic dehydrogenase, two techniques were employed for analysis, namely, the Thunberg technique, using the rate of decolorization of methylene blue in the presence of succinate and nuclei, and the Warburg technique for measuring oxygen consumption caused by the nuclei in the presence of succinate and added cytochrome C. The failure to detect appreciable succinic dehydrogenase by these methods might at first sight appear to conflict with the results recently obtained by Schneider.[34] However, Schneider's nuclei admittedly were contaminated with large granules from the cytoplasm, which contain a relatively high concentration of succinic dehydrogenase,[34] since Schneider's aim was to obtain all of the nuclei, rather than part of the nuclei in a highly purified state. Our nuclei do not appear to contain large granules in significant amounts. Great care has been taken to remove these by centrifuging the nuclei at low speed, and we find that Schneider's remark in the discussion following this paper about the difficulty of microscopic observation of these granules is unfounded as far as we are concerned.

Lately, using nuclei of rat-liver cells obtained by our improved method, we again have failed to demonstrate succinic dehydrogenase by the rate of decolorization of methylene blue in the Thunberg tube, and by the Warburg technique. Apparently then, the absence of appreciable succinic dehydrogenase can be used as one criterion of purity for samples of liver-cell nuclei prepared by mild procedures. In spite of the lack of succinic dehydrogenase activity, cytochrome oxidase was still readily detected, although a reinvestigation of this enzyme from the quantitative standpoint has not yet been made.*

Of all the enzymes studied, alkaline phosphatase was found to be the only one appearing in considerably higher concentration in the nucleus than in the cytoplasm. This result seems to be in agreement with the results obtained by Willmer, using the Gomori technique, which show a high concentration of alkaline phosphatase in the chromosomes.[45]

Some results of enzyme studies of liver tumors made by Greenstein[44] also are shown in TABLE 2. It can be seen from a comparison of these results with the results of studies of the isolated nuclei that there is by no means complete agreement between the two in enzyme pattern. However, it is interesting to notice that the enzymes low in the nuclei, that is, catalase, succinic dehydrogenase, and choline oxidase, also are low in the tumors. Cytochrome C, likewise, appears to be low in the nuclei and in the tumors.

* Since this article was written, a number of quantitative studies have been made of cytochrome oxidase, succinic dehydrogenase, and catalase in nuclei isolated from normal rat-liver cells by our improved method at pH 6.0. In agreement with previous work, succinic dehydrogenase was never found in detectable amounts in the nuclei, although cytochrome oxidase was always found and in concentrations approximating those previously reported. Our previous work with catalase was not confirmed, however, since this enzyme was found in liver- and kidney-cell nuclei in concentrations greater than 50 per cent of the concentrations typical of whole cells. This discrepancy is very likely due to lack of a refrigerated centrifuge when nuclei were first studied in this laboratory. We now have concluded that catalase is an important constituent of liver-cell nuclei. High catalase activities have also been found in kidney-cell nuclei.

It seems probable that too few enzymes have been studied thus far in isolated nuclei to determine for certain whether or not the enzyme pattern of the isolated nuclei resembles the common enzyme pattern of tumors. Furthermore, other kinds of nuclei must be studied before positive conclusions can be drawn.

The enzymes marked in TABLE 2 with an asterisk have been studied in nuclei prepared by our improved method, reference to which has been made previously. A detailed investigation of the enzyme aldolase has been published[46] and details of the work on the other enzymes will appear elsewhere.

In addition to the enzymes appearing in TABLE 2, it has been possible to demonstrate qualitatively the presence of xanthine dehydrogenase, malic dehydrogenase, and a triose phosphate dehydrogenase. Presumably, the triose phosphate dehydrogenase is 3-phosphoglyceraldehyde dehydrogenase, since it acts on synthetic dl-3-phosphoglyceraldehyde (very kindly supplied by Drs. H. O. L. Fischer and E. Baer) as well as the triose phosphate mixture produced by the action of aldolase on fructose diphosphate. Von Euler believes that thymonucleodepolymerase also is present in isolated nuclei,[29, 30] although his evidence does not appear to be conclusive.

Coenzyme I and flavine adenine dinucleotide both are detectable in nuclei prepared from liver cells by the method of Dounce,[2] but both are present in insufficient quantity fully to activate apoenzymes requiring them. For instance, coenzyme I must be added to obtain full activity of lactic dehydrogenase and malic dehydrogenase, and flavine adenine dinucleotide must be added to obtain full activity of d-amino oxidase.[22] In the case of coenzyme I, it is very possible that most of this material is extracted and lost during the washing of the nuclei with water. It is not so certain however that this happens with flavine adenine dinucleotide, since in this case the combination of the prosthetic group with the apoenzyme (at least for d-amino oxidase) appears to be more stable than in the case of coenzyme I.

It should be noticed that in the original determinations of lactic dehydrogenase in isolated nuclei carried out by Dounce[2] and, also, in the determinations carried out by von Euler,[29] no diaphorase was added, although the methylene blue technique was employed. This was unfortunate, since the limiting factor in the reaction could easily be a low diaphorase content of nuclei. Von Euler found a very low activity of lactic dehydrogenase in nuclei, but this method of preparation involved a short exposure of the nuclei at zero degrees C. to a pH of 4.0. This may have injured the apoenzyme or diaphorase or both. We have, however, recently found an apoenzyme concentration in the isolated nuclei of about 40 per cent that of whole tissue in experiments in which the system was completely activated with diaphorase. The activity is, thus, very marked and the decolorization of methylene blue takes place in five minutes or less using 0.1 ml. portions of suspended nuclei. Since the time of decolorization of methylene blue is much longer without the addition of diaphorase, the activity of the nuclei in respect to diaphorase is quite low. The very prolonged decolorization times recorded by von Euler must have been due to a lack of diaphorase or to the effect of the low pH which he employed.

A question of importance concerning the enzyme systems in cell nuclei appears to us to be whether the isolated nuclei can cause glycolysis. This question is now being investigated. Because of the probable removal of all intermediate substrates from the nuclei during their preparation (if indeed these substrates were there originally in appreciable concentrations), a long induction period would be expected before the formation of lactic acid could occur. Appreciable reaction velocity at each step is dependent upon the establishment of reasonable substrate concentration at each step. If one starts with fructose diphosphate, for example, the first two or three steps of glycolysis should be easily measurable in a time interval of a few hours, but subsequent steps probably would require many hours to become measurable unless very large quantities of nuclei were used. Moreover, if the total glycolytic system were to be measured, it would be necessary to add all coenzymes and metallic activators known to function in glycolysis, such as coenzyme I, adenosine diphosphate, magnesium, and possibly calcium and potassium.

Owing to the considerable length of time which probably would be required for the establishment of appreciable amounts of all intermediates in the glycolysis system, it does not, therefore, seem surprising that we have obtained no appreciable formation of lactic acid from the action of isolated liver-cell nuclei on fructose diphosphate, even in the presence of adenosine diphosphate, coenzyme I, magnesium, calcium, and potassium. Even if small amounts of lactic acid had been formed, however, its identification would have been difficult in the presence of larger amounts of earlier intermediates of the glycolysis system.

Because of the difficulties just mentioned, it is perhaps preferable to attack the problem of determining whether glycolysis can occur in the liver-cell nucleus by the more laborious method of determining the enzymes of the glycolysis system singly. Thus far, as has been indicated previously, we have found that aldolase, enolase, lactic dehydrogenase, phosphorylase, and triose phosphate dehydrogenase are present in the nuclei isolated from rat liver by our improved method. Aldolase has been investigated in detail by means of a new colorimetric method.[46]

Discussion of the Validity of Enzyme Studies on Nuclei Isolated by Aqueous Extraction Procedures

It has been argued, on the one hand by Barron,[47] that only positive results are of significance in studies of enzymes in nuclei isolated by the method of Dounce, and, on the other hand by Schneider (see discussion following this paper), that the enzymes cytochrome oxidase and succinic dehydrogenase appearing in his nuclei may well be caused by contamination of the nuclei by large granules. Dr. Claude, in the discussion following the presentation of this work, also argued that the enzymes found in the nuclei might have come from a contamination of the nuclei with large granules, since he has found many enzymes to appear in higher concentrations in the large granules than elsewhere.

The question of contamination of our nuclei with large cytoplasmic

granules has been dealt with, when it was stated that such contamination apparently did not occur to an appreciable extent. There is still another possibility for contamination, however, which is not so easily ruled out. This is the possibility that enzymes might be adsorbed from solution on the surfaces of the nuclei. One argument against this supposition, which already has been given,[2] is that the enzyme catalase, which ordinarily is very easy to adsorb and which has an isoelectric point near 6.0 (its isoelectric point is 5.7), is found only in very low concentrations in the nuclei. Another argument is that washing the nuclei in physiological saline extracts appreciable quantities of some enzymes and apparently causes the nuclei to shrink from loss of protein,[2] but the same enzymes are still found in the washed nuclei. It seems likely that enzymes adsorbed on the surface of the nuclei would be quite easily removed by this procedure. As a matter of fact, a small amount of hemoglobin which apparently is adsorbed in the nuclei is quite well removed by this procedure.[2]

We have recently completed experiments with arginase which indicate that this enzyme is not appreciably adsorbed by isolated nuclei.[48] Also, we have found that the phosphorylase activity of isolated rat liver-cell nuclei is markedly enhanced if the nuclei are broken by grinding in a ground glass homogenizer. If the phosphorylase were simply adsorbed on the nuclear surfaces, no such grinding should be necessary in determining its activity. Grinding is necessary, presumably, because the glycogen used as substrate has such a high molecular weight that it penetrates the isolated nuclei quite slowly.

Finally, such a variety of enzymes have been found in the nuclei in concentrations comparable to their concentrations in cytoplasm that it seems hard to account for so much enzymatic material if it is merely adsorbed on the nuclear surfaces.

It would be difficult to deal with the question of the washing out of enzymes from the nuclei during their preparation were it not for the fact that many enzymes have been found in the nuclei in reasonably high concentrations. It appears unlikely that the enzyme proteins are easily removed from the nuclei by washing with water. Returning to the case of catalase, it appears unlikely that this enzyme should be very completely washed out, since it has a molecular weight of 250,000 and is a globulin, while aldolase, for example, which probably has a much lower molecular weight,[49] remains in quite high concentration. The experiments with isotonic saline also point to the difficulty of removing enzymes from the nuclei by washing.

It has been argued by Dr. Claude in this monograph that it is more important to determine the total amount of enzyme present in various cellular fractions than to determine the concentration of enzyme in a particular fraction. Schneider has stressed the importance of accounting for all of a particular enzyme present in the cell by summing the amounts found in the various fractions.[34]

We have approached the problem from exactly the opposite point of view. Our intention has been to obtain nuclei in as pure a state as possible

and then to determine the enzyme concentration in the isolated nuclei. In the liver cell, as has been stated, the nucleus comprises only about 8 per cent of the cell volume.[20] Therefore, one could not expect to find more than 8 to 10 per cent of the total amount of a given cellular enzyme in the nucleus, assuming equal concentrations of enzyme in the nucleus and the cytoplasm. To find a quantity of enzyme in the nucleus equal to the quantity in the cytoplasm, the concentration of enzyme in the nucleus would have to be many times greater than the concentration in cytoplasm. The argument has been offered that it might be best to ignore the presence of an enzyme in a cell fraction unless the major amount of the enzyme in the cell is carried in this fraction. If this conclusion were applied to non-enzymatic constituents, we would be forced to state that the nucleus contained no protein, other than histone, no phospholipid or lipid, and no ribonucleic acid in the nucleolus. The only constituents remaining would be desoxyribonucleic acid and histone, which are not known to occur in appreciable amounts in the cytoplasm. It appears to us that to draw such a conclusion would be as erroneous as to argue that the nucleus itself should be ignored since it comprises such a small fraction of the volume of the cell.

In a very interesting paper by A. M. Clark,[50] it is demonstrated that digestion of food by *Amoeba proteus* is dependent upon the presence of the nucleus, and the suggestion is made that the digestive enzymes are synthesized as zymogens in the nucleus and become activated only after passing out into the cytoplasm. In addition, the statement is made that a peroxidase which occurs in the nucleus and cytoplasm of the amoeba, and which tends to accumulate at the surface of food vacuoles, also is dependent upon the nucleus for its existence, since in enucleated amoebas it does not appear in the cytoplasm following the ingestion of food.

The work of Duryee which is outlined in this monograph also indicates that at least in special cases the nucleus must carry out some sort of metabolism in the resting stage. Furthermore, it appears to be generally true that just before cell division some metabolism must occur in the nucleus to account for the thickening of the chromosomes and changes in staining properties which can be observed. Other evidence to support this statement will be found in Clark[50] and in the paper of Stern already mentioned.[40] Metabolism of any sort thus far known requires the presence of enzymes. Thus it appears logical that enzymes should occur in the nucleus.

Concluding Remarks

In this paper, we have tried to present a general picture of research concerned with enzymes in the cell nucleus and to present a point of view based on the relatively meager amount of information thus far available, which, it is hoped, will encourage other workers, including enzymologists, to enter this field of research. It appears that this will be necessary if results are to be obtained in a reasonably short time. There is a need for critical experiments designed to check the validity of work already accomplished.

It appears to us that, in selected cases, it might be possible to obtain sufficient nuclei by microdissection to permit enzyme determinations to be carried out. For instance, the Cartesian diver technique might be used with certain respiratory enzymes. The use of microdissection should provide nuclei in a condition very closely resembling their state in the whole cell, and would, therefore, be invaluable in checking the results obtained from nuclei prepared by large-scale methods. Microdissection already has been used to obtain nuclei in which the enzyme catalase was studied. This work, reported by Bundling,[51] shows that catalase is low in the nucleus of salivary gland cells of *Chironomus*, compared to its concentration in cytoplasm. Microdissection studies might also be useful in solving the problem of the state of nucleic acid in isolated nuclei, which has been outlined briefly by Dounce.[24] The use of histochemical enzyme tests will, also, be most appropriate in checking results, if a sufficiently wide range of tests can be developed.

We are of the opinion that work already done shows that many enzymes can be found in a cell nucleus in significant concentrations, and that now the enzyme patterns of nuclei, isolated from as many tissues as possible, must be determined. Before this task is accomplished, however, much work remains to be done in establishing various satisfactory methods for obtaining nuclei suitable for enzyme work from tissue other than liver, kidney, or pancreas, or in finding a method free from obvious drawbacks which can be applied to all tissues.

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Discussion of the Paper

Dr. Walter C. Schneider (*Rockefeller Institute for Medical Research, New York, N. Y.*): In studies of the enzyme distribution in tissue fractions, two factors must always be considered: (1) the specific enzyme activity of the fractions and of the whole tissue and (2) the proportion of the total enzyme activity present in the whole tissue which is associated with each tissue fraction.

Dr. Dounce's results with cytochrome oxidase indicate that isolated rat liver nuclei had a specific activity which was 50 to 60 per cent as great as the specific activity of whole rat liver. In our own experiments (J. Biol. Chem. **165:** 585, 1946), rat liver cells were broken in water and separated by centrifugation into a nuclear fraction, a large granule or mitochondria fraction, and an unfractionated residue. The nuclear fraction contained all of the nuclei present in the original tissue as shown both by microscopic examination and chemical analyses for desoxyribose nucleic acid. The nuclear fraction was, however, contaminated by large granules and the nuclei had clumped in large masses. The following cytochrome oxidase values were obtained (the specific activities are expressed as cu. mm. O_2 consumed per mg. dry material per hour): rat liver, 197; nuclear fraction, 100; large granule fraction, 786; unfractionated residue, 37.8. The data confirm the results of Dr. Dounce, since the specific activity of the nuclear fraction was about 50 per cent as great as that of the original rat liver.

On the other hand, the data clearly show that the large granule fraction

was the only fraction in which the specific activity was higher than in the liver tissue. In view of the high specific activity of the large granule fraction and of the observation that the nuclear fraction was contaminated with large granules, it seems highly probable that the cytochrome oxidase activity observed to be associated with the nuclear fraction was due to contamination by large granules. This is even more clearly indicated by the data on the proportion of the total activity present in the rat liver which was found in each of the fractions: nuclear fraction, 5.4 per cent; large granule fraction, 74 per cent; unfractionated residue, 14.6 per cent. Thus, although the specific activity of the nuclear fraction was about one half as great as the specific activity of the original rat liver, the total amount of enzyme activity associated with this fraction represented only 5.4 per cent of the total enzyme activity present in the rat liver.

It is impossible to tell whether the cytochrome oxidase activity which Dr. Dounce observed to be associated with isolated rat liver nuclei could be explained in terms of contamination with large granules. In this connection, it might be mentioned that large granules in hypotonic solutions are very poorly defined in the light microscope and that their presence might easily be overlooked unless special precautions were taken. Dr. Dounce has also found that succinic dehydrogenase was present only in traces in isolated rat liver nuclei. Our own results indicated that the ratio of succinic dehydrogenase activity to cytochrome oxidase activity was essentially the same in the liver fractions as in the whole liver. Since the succinic dehydrogenase activity of rat liver is only about one-third as great as the cytochrome oxidase activity, the possibility that insufficient amounts of nuclei were used by Dr. Dounce to detect succinic dehydrogenase activity must be considered.

It should also be pointed out that, in our own experiments, over 90 per cent of the total activity of each of three enzymes studied, present in the original liver tissue, was recovered in the tissue fractions. Thus, there was no possibility that unknown co-factors necessary for the enzyme assays and present in the whole liver tissue were lost in the preparation of the tissue fractions. The need for determining the specific activity and the total activity of the enzyme in the whole tissue and in each of the tissue fractions cannot be emphasized too strongly, for only in this manner is it possible to demonstrate that the techniques of enzyme assay are valid and to assess the significance of the specific activity of an enzyme in a given tissue fraction.

Dr. A. L. Dounce: As has been emphasized in the paper, we believe that, if one wishes to study the composition of nuclei, the first step should be to obtain the nuclei in as pure condition as possible. Recovery of all nuclei present, or of 90 per cent of a constituent present in the cell, appears somewhat irrelevant in view of the relatively small percentage volume occupied by the nucleus in the case of the liver cell. We believe that other methods, such as microdissection and histochemical techniques, should be employed to check results obtained in large-scale experiments on isolated nuclei.

Dr. Jean Brachet (*Department of Zoology, University of Pennsylvania, Philadelphia, Pa.*): Dr. Dounce rightly emphasized the need to compare

the enzymatic constitution of nuclei isolated by the method he used and by microdissection. A few years ago, I made a study of some of the enzymes present in the isolated germinal vesicle of the frog oocyte as compared with the cytoplasm. No respiratory enzymes have been studied so far, but dipeptidase, esterase, alkaline phosphatase, ribonuclease, and arginase were all found to be present in concentrations lower in the nucleus than in the cytoplasm.

It is noteworthy that, according to Duspiva, there is a loss of dipeptidase from the nucleus into the surrounding physiological saline solution which starts as soon as the germinal vesicle has been isolated. This finding, indicating possible loss of enzymes from the nuclei after their isolation, should be kept in mind. As regards the low content of alkaline phosphatase of the germinal vesicle compared to the high concentration of the same enzyme in other nuclei, as found by Dr. Dounce and many other workers using Dr. Gomori's technique, it may be said that the germinal vesicle is very poor in chromatin and rich in nuclear sap, and we know, of course, that the Gomori test is strongly positive in the chromatin and in the nucleoli only.

Dr. A. L. Dounce: It is much to be hoped that the work mentioned by Dr. Brachet will shortly be published with details as to methods employed and results obtained on the percentage concentrations of the enzymes in the nuclei as compared with their concentrations in cytoplasm.

We have found that physiological saline tends to extract enzymes from isolated nuclei to a considerably greater extent than does distilled water, as mentioned in the paper.

THE FUNCTION OF CELL INCLUSIONS IN THE METABOLISM OF *CHAOS CHAOS*

By Heinz Holter

Cytochemical Department, Carlsberg Laboratorium. 8, Gl. Carlsbergvej. Copenhagen, Valby. Denmark.

The starting point for the present studies consisted of a series of observations on the fate of the visible cytoplasmic inclusion bodies in the giant ameba, *Chaos chaos*. These inclusion bodies, shown in FIGURE 1, are: the crystals, the heavy spherical or refractive bodies, the fat droplets, and the food vacuoles.

Of the four inclusion bodies under consideration, two are enclosed within vacuoles, namely, the food balls and the crystals. The physiological function of the food balls is obvious. Regarding the other three, however, opinions have differed, but due to the work of Mast and his co-workers,[1] they are most commonly regarded as reserve food. According to this hypothesis, the contents of the food vacuoles are transformed within the vacuoles into crystals, *etc.* Then the food vacuoles divide into smaller vacuoles, thus spreading their contents over the whole of the ameba's cytoplasm. The non-vacuolized, refractive bodies and the fat droplets disintegrate and leave the food vacuoles by diffusion to be regenerated later in the cytoplasm. The food reserve, so created and distributed, is to be consumed during starvation.

Andresen and the author were interested in the starvation metabolism of *Chaos chaos* and, in order to study the role of the various microscopically visible inclusion bodies, we repeatedly counted their numbers in starving amebas[2] and determined at the same time the decrease in the ameba's volume induced by starvation.[3] Details concerning the technique of these measurements need not be discussed. The result of one typical experiment is shown in FIGURE 2. It will be noted that the concentration of the crystals observed in this instance did not decrease, but actually increased during starvation.

By taking into account the simultaneous change in the total volume of the starving amebas and the total number of crystals in the cytoplasm, rather than their concentration in a microscopic field, one finds that this number remained approximately constant (dotted lines in FIGURE 2). Similar results were obtained with the heavy spherical or refractive bodies.

Regarding the fat droplets, however, the individual variations between single amebas were so great and the results of our counts so erratic that we were unable to draw any unambiguous conclusion except that they did not disappear consistently and gradually during starvation. Concerning the crystals and heavy spherical bodies, on the other hand, one may feel justified in doubting the assumption that they constitute the ameba's reserve food, since it is obvious that they are not utilized during starvation to any appreciable extent.

Before an alternative hypothesis is offered, a few other observations should be mentioned. One concerns the fate of the food vacuoles. If large num-

bers of paramecia are fed to amebas which were slightly starved and therefore did not contain food remnants, the amebas produce many easily recognizable food vacuoles. The total number of such vacuoles formed within each ameba may be counted. Within a few hours after ingestion, the paramecia have been transformed into compact spherical balls. During the next day or two, these vacuoles become smaller and still more compact without ever being broken up and distributed into vacuoles which could compare with the crystal vacuoles in size or number. During the first 48

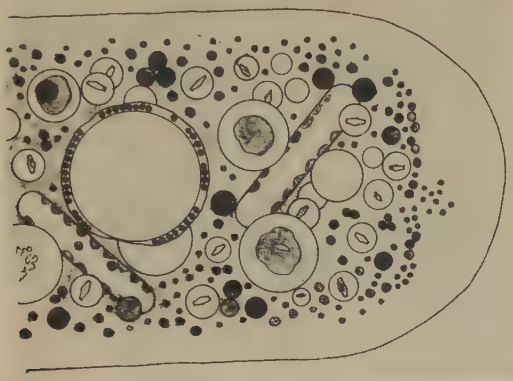

FIGURE 1. Cellular inclusions in *Chaos chaos*. Tip of pseudopodium showing two rod-shaped nuclei, one large contractile vacuole (coated with mitochondria), four food vacuoles, and numerous crystal-containing vacuoles. Refractive bodies (empty circles) and fat droplets (solid circles) are equal in size to the crystal-containing vacuoles. In addition, there are numerous smaller cytoplasmic inclusions.

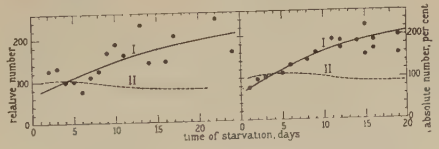

FIGURE 2. Number of crystals in relation to starvation. Curve I. Number of crystals (longer than 1 micron) per counting volume. Curve II. Total number of crystals in entire ameba. Data based on two independent series.

hours, at 22°C, after ingestion, practically all these food balls are defecated and, since they remain recognizable for some time after defecation, one is able to determine that the number of egested balls corresponds to the number of food vacuoles formed.

Another observation concerns the phenomenon of vacuole coalescence,[2, 4] which consists of a fusion of various types of permanent vacuoles other than the contractile vacuoles. This fusion is preceded by a period of agglutination that leads to a mixture of the vacuolar contents (FIGURES 3 and 4). Similar processes may also include the refractive bodies which, in this respect, behave like vacuoles.

The details of all observations cannot be reported here. The collected evidence, however, suggested the following hypothesis: in the food vacuoles,

there is a progressive breakdown of the food balls and an absorption of the soluble degradation products through the vacuolar wall. Physiologically, the food vacuoles correspond to the intestinal tract of the metazoa, since

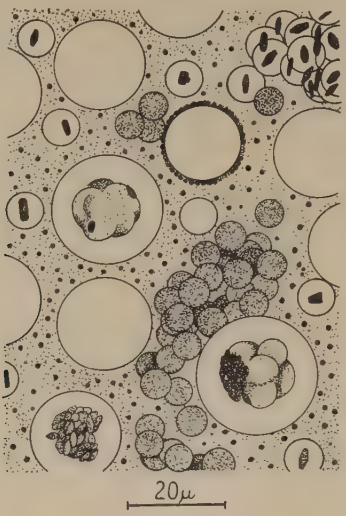

FIGURE 3. Cytoplasmic inclusions in 18-day starving ameba, four days before death. A group of agglutinated crystal vacuoles is shown in upper right corner. Below them, and to the left, is shown a contractile vacuole coated with mitochondria. At its side are three agglutinated heavy spherical bodies. Below these is shown a vacuole with several coalesced spherical bodies and two crystals. Another similar vacuole, but with a cap of crystals on the spherical bodies, is shown at the lower right corner. The moruloid mass to the left represents a group of partially agglutinated, heavy spherical bodies. At the lower left corner is a vacuole containing a concretion of crystals. In addition, the cytoplasm contained numerous empty vacuoles, uncoated by mitochondria.

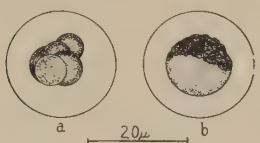

FIGURE 4. Typical coalescence vacuoles: (a) an early coalescence vacuole containing five heavy, spherical bodies; (b) an older coalescence vacuole with numerous crystals forming a cap on a structureless body which may have developed from the coalescence of heavy, spherical bodies.

the vacuolar wall constitutes the inner surface of the ameba and the food substances do not, strictly speaking, enter the cytoplasm of the ameba unless they diffuse through the vacuolar membrane.

The formation of crystals, heavy spherical bodies, and fat droplets is

assumed to occur in the cytoplasm, and, if any of these cytoplasmic inclusions were observed in the true food vacuoles, they probably were formed by vacuole coalescence and not by the primary transformation of food balls. In some respects, the coalescence of vacuoles gives the impression of being one step in a sequence of processes leading to the expulsion of formed excretory products. With crystals, the first step in this process would be the formation of a vacuolar membrane around the crystalline particles. As soon as this is formed, the contents of the vacuoles are already outside the cytoplasm. The next step is the collection of larger lumps of refuse by vacuole coalescence. The final step is egestion (FIGURE 5).

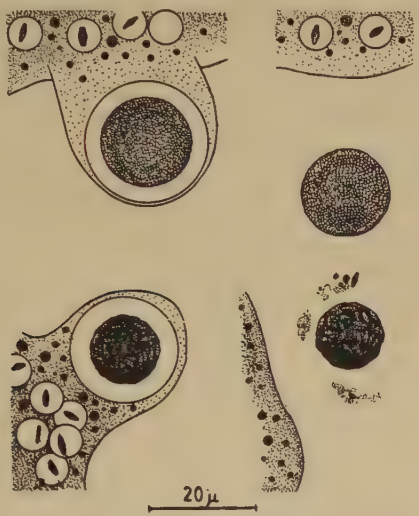

FIGURE 5. Two examples of egestion. Upper group: Egestion of food vacuole. Lower group: Egestion of a druse of crystals. The left figures show the bodies in the tip of a short pseudopodium of hyaline cytoplasm. Mitochondria, fat globules, and crystal vacuoles are present at the base of the pseudopodium. The right figures show the expelled objects lying free in the medium. Note small amount of extruded cytoplasm surrounding the crystal druse.

The defecation of crystal druses formed by the coalescence of crystal vacuoles has been frequently observed.

In the starving ameba, the coalescence phenomenon seems to get out of hand, and huge concretions of crystals, which one may observe during the later stages of starvation, are not defecated. These must, therefore, be regarded as pathological.

The results of the counting experiments indicate rather strongly, in our opinion, that whatever the physiological function of the crystals and refractive bodies may be, it is hardly that of reserve food.

Zeuthen and the author,[12] therefore, attempted to study the starvation metabolism of these amebas by suitable chemical means. A very promising method, based on the Cartesian diver technique, was developed by Zeuthen.[5]

It is the application of the diver principle to the weighing of small objects under water. The "diver balance," as Zeuthen calls it, is shown in FIGURE 6.

Regarding the principles and previous applications of Linderstrøm-Lang's Cartesian diver,[6] reference must be made to the literature.[7] Only the diver balance can be described. It consists of a small glass flask with a very long capillary tail. The wall and bore of the latter are much thinner than shown in FIGURE 6. The body of the flask contains an air bubble which makes it float in an inverted position. The capillary tail permits a rapid adjustment of the gas volume according to the applied pressure while, at the same time, minimizing the loss of gas by diffusion into the surrounding medium. At the top of the inverted flask is placed a cup of polystyrene, which, because of its low density, does not make the diver top-heavy. The

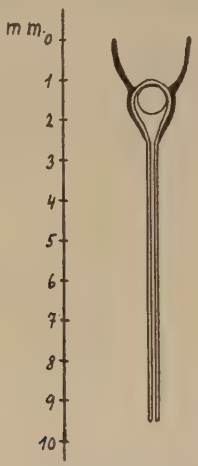

FIGURE 6. Diagram of the Cartesian diver balance. Note the cup of polystyrene mounted on the inverted capillary flask.

diver balance floats in a suitable medium consisting of water or balanced salt solutions, and its equilibrium pressure can be determined with an accuracy of about one or two mm. of water.

Weighing is performed by determining the equilibrium pressure of the "empty balance," then pipetting into the cup the object to be weighed and determining the equilibrium pressure of the "loaded balance." The resulting difference is the so-called "reduced weight" of the object, that is to say, its weight minus the weight of the volume of water displaced. These values alone are very useful reference quantities in biological work, as they provide a measure of the non-aqueous components of the cytoplasm investigated.

If necessary, the absolute weight can be readily determined. This is done by measuring the reduced weight in two media of different densities, one of them being, for instance, a starch solution, the other, water. From the two reduced weights, the absolute weight, the specific gravity, and, accordingly, the volume of the object can be calculated.

In its present dimensions, the diver balance permits the determination of reduced weights in the order of a few gamma with an accuracy of ± 2 per cent. The chief asset of the weighing method is, of course, that it does not injure the object. In our experiments, the individual amebas could be weighed over and over again, and between weighings they could be used for other purposes, *e. g.*, for determining the oxygen consumption in the ordinary micro-respirometer diver.

FIGURE 7 shows the course of such an experiment for four individual amebas. The abscissa in all examples gives the time of starvation in days. The curves of the lower chart give the reduced weights in gammas. At the time intervals marked by dotted lines, the oxygen consumption of the same animals was measured in standard divers, and the middle chart gives

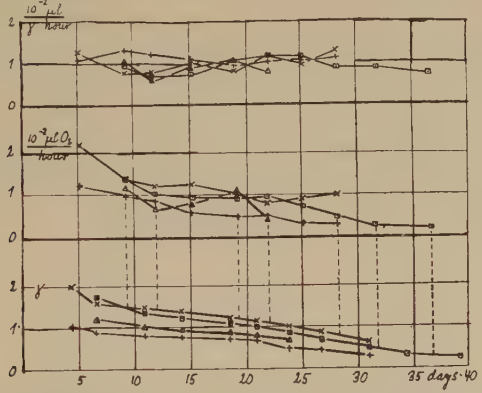

FIGURE 7. Changes in reduced weights and respiratory rates of four individual amebas during starvation. Upper curves represent oxygen consumption in microliters per hour per gamma of reduced weight; middle curves show oxygen consumption per hour; and lower curves give changes in reduced weights in gammas. Days of starvation given in abscissa.

the respiratory rates in micro-liters of oxygen consumed per hour. The upper chart is the ratio between the two sets of values. These ratios are practically constant throughout the experiment, thereby indicating that the respiratory rates depend roughly on the total amounts of cytoplasm, as was to be expected from previous experience.

How can such data elucidate the metabolism of starving amebas? It is obvious that the changes in reduced weight must result from the disappearance of substances with a specific gravity that differs from water. The following densities are assumed for the non-aqueous components of the ameba's cytoplasm:

Proteins	1.35
Carbohydrates	1.55
Fats and lipids	1.0
Salts	2.1

The values, of course, are based on rough estimates, but they give a general idea of the magnitudes involved. The bulk of the cell's substance undoubtedly consists of proteins. Regarding the lipid density, this is based

on the following values: 0.95 for fats, and ca. 1.03 for compound lipids. There are reasons to believe that the amounts of phospholipids and fats are essentially identical. The average density of lipids in the cell, therefore, must be close to 1.0.

With regard to salt content, we have assumed that the ameba regulates its total salt concentration to a constant value, at least as long as its condition can be regarded as non-pathological. This means that, during starvation, salts are gradually excreted in rough proportion to the amount of protein metabolized. If we make this assumption and if we further assume the total value of the normal osmotic concentration to be about equivalent to 0.025M NaCl,[8] the salt loss can be accounted for by adopting a slightly higher value for the specific gravity and reduced weight of the protein. Numerically, this works out to an increment of 0.02, thus bringing the specific gravity of the proteins to 1.37. Fortunately, this is a rather small correction. On the basis of these assumptions, the factor converting the observed change in reduced weight into absolute weights of protein is 3.7.

With regard to carbohydrates, the corresponding factor would be 2.9. The carbohydrate content of the ameba, however, is very small. For reasons to be explained later, it is possible to disregard completely the possible loss of carbohydrates during starvation. A loss of lipids cannot be measured, since their average density is the same as the water in which the diver balance is floating.

If these assumptions are accepted, the evaluation of the experiments becomes simple. We know the ameba's oxygen consumption and the amount of protein that has disappeared within a given interval of time. We know, from studies on other tissues and organisms, the amount of oxygen needed to combust the protein which has disappeared. If the total oxygen consumption is larger than these values (disregarding the carbohydrates), the difference must have been used for the combustion of substances which do not influence the reduced weight, in other words, for the combustion of lipids.

A close inspection of the curves in FIGURE 7 shows that the decrease in reduced weight has a tendency to slow up after the first few days of starvation. This tendency is brought out more clearly in FIGURE 8, where the decrease in reduced weight is plotted daily in per cent of the preceding volume.

In the middle of the starvation period, we may expect that the combustion of lipids will be highest compared with the combustion of heavier cytoplasmic components. Of the two examples considered in TABLE 1, Ameba No. 5 was selected for high lipid turnover. This ameba was characterized by a normal respiratory rate but rather small changes in reduced weight over a 10-day period. Ameba No. 11 was followed throughout the whole period of starvation, beginning at the time where the greatest part of the primary food vacuoles had disappeared.

The data in TABLE 1 show, in both instances, considerable differences between the actually observed oxygen consumption and the oxygen con-

sumption accounted for by the change in reduced weight, assuming, of course, that these changes were entirely due to the combustion of protein (corresponding oxygen consumption). These differences in oxygen consumption have been called lipid oxygen, and the corresponding amount of combusted lipids in absolute weight was, in Ameba No. 5, nearly the same as the total protein loss in the period considered. Even in Ameba No. 11, where the whole starvation period was included in the calculation, the lipids form a considerable portion of the total substance metabolized.

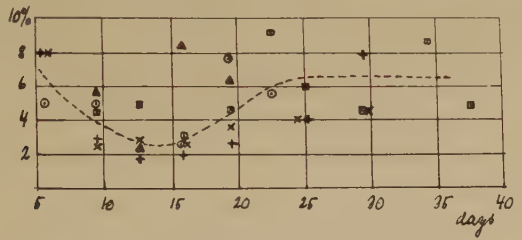

FIGURE 8. Percentage decrease in reduced weight during starvation. Decrease in reduced weight plotted daily in per cent of preceding volume.

TABLE 1

	Ameba no. 5	*Ameba no. 11*
	Days of starvation	
	10 to 20	*4 to 27*
Δ *Reduced weight*	0.14γ	0.59γ*
Corresponding protein	0.52γ	2.18γ*
Corresponding oxygen	0.52λ	2.18λ†
Observed Δ oxygen consumption	1.53λ	3.90λ†
Lipid oxygen	1.01λ	1.72λ†
Corresponding lipid	0.50γ	0.86γ*
*γ Lipid/γ Protein	0.96	0.39
cal. Lipid/cal. Protein	2.2	0.89
Δ *Reduced weight*, 4th day	—	0.98γ*
Absolute weight	—	50.0γ*
Volume	—	0.049λ†
Maximum protein	—	3.6γ (7.2%)*
Minimum lipid	—	0.86γ (1.7%)*

* γ = gamma = 1×10^{-6} gm.
† λ = microliter = 1×10^{-6} liter.

This becomes still more evident if we compare not the absolute amounts of the substances but their energetic equivalents when converted roughly on the basis of factors known from human physiology. On this basis, Ameba No. 5 had, in the period considered, covered two-thirds of its energy requirements by combustion of lipids. In Ameba No. 11, nearly one-half of the total energy utilized during the entire starvation period had been supplied by lipids.

On the basis of the assumptions made, the values for reduced weight and oxygen consumption can also be used for a rough calculation of the initial

protein and lipid content of the ameba cytoplasm prior to starvation. For Ameba No. 11, these calculations gave a maximum value of 7.2 per cent for protein content and a minimum value of 1.7 per cent for lipid content (see TABLE 1).

The protein content thus determined cannot be checked by analysis of the same ameba, but a direct nitrogen determination of 15 amebas in the same state of nutrition, by a micro-Kjeldahl method,[9] gave 5.0 to 7.5 per cent protein, with a mean value of 6.4 per cent. The amount of lipid used by the ameba during the period of observation was 1.7 per cent of its initial wet weight. This, therefore, is the minimum amount of lipid present at the beginning of the experiment and, as pointed out before, it is determined on the assumption that the only other material oxidized is protein. If we substitute carbohydrate, with its higher density, as part of the fuel, the proportion of lipids used becomes still higher. This is why we did not consider the carbohydrates in our previous calculations.

The lipid value is only a minimum in any event, and the actual lipid content must be considerably higher. But even the minimum value is interesting in view of the fact that, in previous determinations of the contents of visible fat in the cytoplasm of *Chaos chaos*,[2] the highest value ever found was 0.5 per cent of the ameba's volume, while the average was much lower. This indicates that the microscopically visible fat droplets do not form the most important metabolic lipid resource of the amebas.

In other amebas that had been treated in the same way as the two examples described here, we have found varying proportions between lipids and heavy substrates combusted, but, in all instances so far analyzed, the invisible cytoplasmic lipids seem to constitute an important source of energy to the starving ameba. In the initial and final periods of starvation, heavy substrates usually dominate, but, in the middle periods, the lipids often play the main role.

In this connection, it should be noted that the period of excessive vacuole coalescence seems to set in when the period of slow decrease of the reduced weight is over, in other words, when the lipid content of the cytoplasm has become low. It is tempting to assume that the two phenomena are connected. It might mean, for instance, that the surface of the vacuolar membranes is dependent on the presence of a certain amount of cytoplasmic lipids.

What independent evidence can be produced to support the rather numerous assumptions made and the perhaps too far-reaching conclusions drawn? The possibility of checking the calculated protein content by direct Kjeldahl analysis has already been mentioned. Determinations of total lipid contents in amebas, according to the method of Schmidt-Nielsen,[10] should also be possible and will be attempted.

Respiratory quotients can be determined in the standard diver.[11, 12] This has been done in several instances, and the R. Q. values obtained so far have ranged between 0.75 and 0.88, which is consistent with the assumption of a mixed combustion of lipids and proteins. Here again, further work is needed.

So far, we have not been able to search the literature exhaustively for similar data on related material. But it ought to be mentioned that Parpart[13] and Hunter[14] found a total lipid content of 5.4 per cent wet weight in the cytoplasm of *Arbacia* eggs, and 77 per cent of the total lipids behaved as if they were cytoplasmatically bound. Heilbrunn[15] has reported on the release of bound lipid from the cytoplasm of *Arbacia* eggs and of *Ameba proteus* induced by the action of ammonium salts.

Finally, the author wishes to point out that the main object of the present paper was not the rather limited field of the metabolism of *Chaos chaos* but, rather, a demonstration of the general usefulness of Zeuthen's diver balance for cytochemical and cytophysiological research.

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CONCLUDING REMARKS

By Robert Chambers
Department of Biology, New York University, New York, N. Y.

A general criticism which can be made of any experimental study for elucidating protoplasmic activities is the fragmentary nature of the conclusions to be drawn from the results achieved. We must always be aware of the necessarily limited view, but not lose hope that other aspects will eventually add with ours toward further revelation. Some of us, even most of us, may be off the main path, but, provided we painstakingly describe the methods and the precise nature of the material we use, we may be certain that at least some fraction of the results will have its value.

One of the pregnant problems of cellular physiology is the nature of the limiting boundary of the protoplasmic body of a cell. Our eyes should be kept open to the possibility of serious error in interpreting any limiting envelope after its removal from the internal cytoplasm.

We should also be on the alert in interpreting observations of isolated constituents of cells. The isolation involves destruction of the cell. In this process many changes occur, which not only affect the visible morphology, but the more subtle chemical and physical structure as well. The nucleus, as it is being isolated, most probably loses or gains materials from the dying cytoplasm. By the same token, products isolated from cells are exposed to cytolytic reactions. When separated, for example, by centrifugation, the formed elements may be different in any given respect from the granules visible in the living cell. Some of the segregated granules may have appeared during cytolysis; others may have lost or assumed properties which they did or did not have in the living cell.

The study of these cellular products has its own value. Their interpretation can be assisted by histochemical reactions within the integrated cell. Here again there is room for question, since most of the histochemical methods at our disposal are used on cells which have undergone the drastic changes of histological fixation. Some chemical observations have been made on structures *in situ* on the living cell. The conclusions drawn from results of the isolation experiments must be in accord with what can be obtained on the living cell.

Micromanipulative studies are of great value for an understanding of physical structure. Those who are experienced with the technique are astonished at the relative lack of damage when a microneedle is inserted into a living cell. Even here it is necessary to appreciate the resulting changes and to draw conclusions only when the changes can be shown to be reversible.

A study of model systems should be encouraged in attempts to reproduce structures which seem to be present in the living cell. An example of this, presented in one of the papers of this symposium, is the study of artificially produced protein fibers. This should be done irrespective of

whether the structures duplicate the more intimate ones in protoplasm. We have learned much from the model systems early developed by Butschli and Ludwig Rhumbler.

I conclude with a story told by Dr. Albert Szent-Gyorgy in one of his recent lectures on muscle contraction. A holy man asked to be given a glimpse of heaven. This plea was accorded him, and, when he looked in, lo! he found himself gazing at the portals of a second heaven. Let us look into our heavens, but we must expect that there will be other heavens still to be looked into.

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